

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 March 2003 (27.03.2003)

PCT

(10) International Publication Number
WO 03/024200 A2

(51) International Patent Classification⁷: **A01H**
(21) International Application Number: **PCT/US02/30195**
(22) International Filing Date:
23 September 2002 (23.09.2002)
(25) Filing Language: **English**
(26) Publication Language: **English**
(30) Priority Data:
60/324,014 21 September 2001 (21.09.2001) **US**
09/992,634 14 November 2001 (14.11.2001) **US**

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (*for all designated States except US*): **AVIGEN-ICS, INC. Georgia Bio-Business Center** [US/US]; 111 Riverbend Road, Athens, GA 30605 (US).

Published:

— *without international search report and to be republished upon receipt of that report*

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): **SUTRAVE, Pramod** [AU/US]; 101 Ashley Circle, Apartment 3, Athens, GA 30605 (US).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(74) Agents: **ANTLER, Adriane, M. et al.; Pennie & Edmonds LLP**, 1155 Avenue of the Americas, New York, NY 10036 (US).

(54) Title: **PRODUCTION OF TRANSGENIC BIRDS USING STAGE X PRIMORDIAL GERM CELLS**

(57) Abstract: The present invention relates to methods for isolating primordial germ cells (PGCs) from the blastoderm of a stage X avian embryo. The present invention further relates to methods for producing a transgenic bird by modifying the isolated PGCs, such that the cells incorporate at least one transgene into their genetic material; transferring the modified PGCs into a suitable recipient, such as a blastoderm of an avian embryo, hatching the embryo; and testing for the presence of the transgene or expression of the protein encoded by the transgene.

WO 03/024200 A2

10

15

20

30

35

molecules. The microinjected fertilized eggs are then transferred to the genital tract of a pseudopregnant female (*e.g.*, Krimpenfort *et al.*, in U.S. Pat. No. 5,175,384).

This widely used technique requires equipment to microinject the eggs *in vitro* and to handle the embryos. Large numbers of fertilized eggs are needed because of the high rate
5 of egg loss due to lysis during microinjection. Moreover, manipulated embryos are less likely to implant and survive in utero. Typically, 300-500 fertilized eggs must be microinjected to produce perhaps three transgenic animals. Consequently, generating large animals with these techniques is prohibitively expensive.

Genetic information also has been transferred to embryos using retroviral vectors
10 (Jaenisch, R., 1976, Proc. Natl. Acad. Sci. USA 73, 1260-1264), but the resulting animals were mosaics with gene insertions at various loci in the genomic nucleic acid of any one animal. The transgenes also were differentially expressed in various tissues of each animal (Jaenisch, R., 1980, Cell 19, 181-188).

Nuclear transfer from cultured cell populations is a further route to genetic
15 modification, whereby donor cells can be sexed, optionally genetically modified, and then selected in culture before their use. The resultant transgenic animal originates from a single transgenic nucleus and mosaics are avoided. The genetic modification is easily transmitted to the offspring. Nuclear transfer from cultured somatic cells also provides a route for directed genetic manipulation of animal species, including the addition or "knock-in" of
20 genes, and the removal or inactivation or "knock-out" of genes or their associated control sequences (Polejaeva *et al.*, 2000, Theriogenology, 53, 117-26). Gene targeting techniques also promise the generation of transgenic animals in which specific genes coding for endogenous proteins have been replaced by exogenous genes such as those coding for human proteins.

25 Although gene targeting techniques combined with nuclear transfer hold tremendous promise for nutritional and medical applications, current approaches suffer from several limitations, including long generation times between the founder animal and production transgenic herds, and extensive husbandry and veterinary costs. It is therefore desirable to use a system where cultured somatic cells for nuclear transfer are more efficiently
30 employed.

One system that holds potential is the avian reproductive system. The production of an avian egg begins with formation of a large yolk in the ovary of the hen. The unfertilized oocyte or ovum is positioned on top of the yolk sac. After ovulation, the ovum passes into the infundibulum of the oviduct where it is fertilized if sperm are present, and then moves
35 into the oviduct magnum that is lined with tubular gland cells. These cells secrete the egg-

white proteins, including ovalbumin, lysozyme, ovomucoid, conalbumin and ovomucin, into the lumen of the magnum where they are deposited onto the avian embryo and yolk.

The hen oviduct offers outstanding potential as a protein bioreactor because of the high levels of protein production, the promise of proper folding and post-translation
5 modification of the target protein, the ease of product recovery, and the shorter developmental period of chickens compared to other potential animal species. As a result, efforts have been made to create transgenic chickens expressing heterologous proteins in the oviduct by means of microinjection of DNA (PCT Publication WO 97/47739).

Bosselman *et al.* in U.S. Patent No. 5,162,215 describe a method for introducing a
10 replication-defective retroviral vector into a pluripotent stem cell of an unincubated chick embryo, and further describe chimeric chickens whose cells express a heterologous vector nucleic acid sequence. However, the percentage of G₁ transgenic offspring (progeny from vector-positive male G₀ birds) was low and varied between 1% and approximately 8%. DNA injection into avian eggs has so far led to poor and unstable transgene integration
15 (Sang and Perry, 1989, Mol. Reprod. Dev., 1: 98-106) and Naito *et al.*, 1994, Mol. Reprod. Dev. 37: 167-71). In addition, the use of viral vectors imposes limitations upon the success of transgenesis, including limited transgene size and potential viral infection of the offspring. The production of transgenic chickens by DNA microinjection can also be both inefficient and time-consuming.

20 Another method for generating transgenics is the stable transfection of male germ cells *in vitro* and their transfer to a recipient testis. PCT Publication WO 87/05325 describes a method of transferring organic and/or inorganic material into sperm or egg cells by using liposomes. Bachiller *et al.*, (1991, Mol. Reprod. Develop. 30: 194-200) used Lipofectin-based liposomes to transfer DNA into mice sperm, and provided evidence that
25 the liposome transfected DNA was overwhelmingly contained within the sperm's nucleus, although no transgenic mice could be produced by this technique. Nakanishi and Iritani (1993, Mol. Reprod. Develop. 36: 258-261) used Lipofectin-based liposomes to associate heterologous DNA with chicken sperm, which were in turn used to artificially inseminate hens. Although the heterologous DNA was detectable in many of the resultant fertilized
30 eggs, there was no evidence of genomic integration of the heterologous DNA either in the DNA-liposome treated sperm or in the resultant chicks.

Primordial germ cells (PGCs) give rise to embryonic germ cells and ultimately the gametes of mature adults. Late development stage PGCs from stage 27 chick embryos have been isolated (Chang *et al.*, 1997, Cell Biol. Int. 21: 495-499). PGCs have also been
35 isolated from stage XIII to XIV embryos by insertion of a micropipette into the dorsal aorta

of the embryo and extraction of blood containing PGCs. (Naito *et al.*, 1994, Mol. Reprod. Dev 39: 153-171; Ponce de Leon *et al.* in U.S. Patent No. 6,156,569). Earlier stage PGCs appearing at stages VII – IX (Karagene *et al.*, 1996, Dev. Genet. 19: 290-301) are fewer in number than stage XIII – XXVII PGCs and harder to extract. Cell surface markers typical of PGCs, like SSEA-1 and EMA-1, appear only at stage X of differentiation. Isolation of these cells has required culturing on feeder cell layers to allow proliferation and enrichment of the population, which is both time consuming and technically demanding. Once available, however, PGCs offer a means of generating transgenic animals having heterologous genes in the gamete cells. These animals can be interbred to create homozygous animals with heterologous expression and protein production in some or all tissues.

Once a transgenic animal line has been created, the protein expressed from the integrated transgene can be produced in quantity and bears post-translational modifications such as glycosylation that can be necessary for functionality. The exogenous protein can be produced in the white of an avian egg, from which it can be readily purified. The economic advantage of breeding flocks of transgenic birds laying eggs expressing exogenous proteins is significant when compared to more traditional animals, such as cows and goats, producing heterologous protein in milk.

There remains a need for methods of generating transgenic chickens in which heterologous genes are stably integrated into the genome of transgenic chickens. Citation or identification of any reference in Section 2 of this application is not an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention provides methods for isolating avian stage X primordial germ cells that can be genetically modified and then introduced into the blastoderm of a recipient egg. The recipient embryo is then allowed to develop into a viable chick. The hatchling can be a chimeric bird having at least some germ cells derived from the introduced stage X primordial germ cells. It is further contemplated that isolated primordial germ cells can be transfected *ex vivo* so that the chimeric bird can have a population of germ cells that comprise heterologous nucleic acid (*i.e.*, a transgene) therein. The heterologous nucleic acid can encode a polypeptide desired to be produced by a transgenic bird. The resultant chimeric transgenic avian can be used for subsequent breeding to generate transgenic avians that are fully transgenic and have the transgene integrated into their genome.

The invention is based, in part, on Applicant's method of enriching for, preferably isolating, avian stage X primordial germ cells from stage X blastodermal cells from an avian embryo and Applicant's realization of the potential use of the enriched stage X primordial germ cells to achieve avian germ line transmission of an introduced nucleic acid.

5 The present invention provides methods for isolating a population of avian stage X primordial germ cells from a mixed population suspension of dissociated stage X blastodermal cells obtained from an avian embryo. Stromal cells are sedimented from the suspension by gravity, and the population of cells thereby enriched in stage X primordial germ cells is then isolated from the residual medium supernatant. The avian embryo can be
10 from any bird including, for example, chicken, turkey, quail, pheasant, duck, goose and ratite. In one embodiment of the method of the present invention, the avian embryo is a chicken embryo.

The methods of the present invention result in isolated cell populations of stage X primordial germ cells that are substantially free of stromal blastoderm cells, have alkaline
15 phosphatase activity, and are positive for Periodic Acid Schiff staining (which staining distinguishes them from stromal cells).

The present invention also provides methods for generating an avian having a heterologous germ cell having an exogenous or heterologous nucleic acid or different genetic background, said method comprising: isolating a population of cells enriched in
20 stage X primordial germ cells; microinjecting this population of cells into a recipient embryo of an avian egg; and allowing the recipient embryo to hatch as a chick having a heterologous germ cell therein. A further embodiment of the method for generating a transgenic avian also comprises the step of allowing the chick having the heterologous germ cell to develop to an adult bird, that can be a chimera, and which has at least some germ
25 cells that are heterologous germ cells.

The present invention further provides methods for the production of transgenic avians capable of expressing a heterologous polypeptide, comprising isolating a population of cells enriched in stage X primordial germ cells; transfecting the isolated stage X primordial germ cells by delivering a heterologous nucleic acid thereto; and then
30 microinjecting the transfected avian stage X primordial germ cells into a recipient embryo of an avian egg. The recipient embryo is allowed to hatch as a chick and mature into an adult bird having cells derived from, *i.e.*, containing the genome of, the transfected germ cell therein. The adult bird having the heterologous transfected germ cells can be interbred with, or its sperm used to artificially inseminate, a second adult bird thereby producing
35 transgenic progeny heterozygous for the heterologous nucleic acid. Breeding the

heterozygous transgenic progeny bird with a second heterozygous transgenic progeny bird can generate a transgenic progeny bird that is homozygous for the heterologous nucleic acid.

In one embodiment of the methods of the present invention, a heterologous polypeptide encoded by the heterologous nucleic acid is produced by the first heterozygous transgenic bird, the second heterozygous transgenic bird, the homozygous transgenic bird, or any progeny thereof.

In various embodiments of the methods of the present invention for the production of a transgenic avian capable of producing a heterologous polypeptide, the heterologous polypeptide is in the serum of the transgenic bird. In still another embodiment of the present invention, the heterologous polypeptide is delivered to the white of a developing avian egg produced by the transgenic bird.

In other embodiments of the methods of the present invention, for the production of a transgenic avian capable of producing a heterologous polypeptide, the heterologous nucleic acid comprises an expression cassette having a promoter, a transcription termination sequence, and a polypeptide-encoding sequence. In one embodiment of the present invention, the expression cassette comprises a transcription unit encoding a first heterologous polypeptide, and optionally a second heterologous polypeptide, operably linked to an avian specific transcription promoter, a transcription terminator, and optionally an internal ribosome entry site (IRES). In another embodiment of the present invention, the transgenic avian expresses a first and a second transgene encoding a first and a second heterologous polypeptide. The method further comprises the step of combining the first and second heterologous polypeptides, thereby forming a multimeric protein.

In one embodiment of the methods of the present invention, the heterologous polypeptide is selected from the group consisting of a cytokine, erythropoietin, a hormone, an enzyme, a structural protein, and an immunoglobulin. The cytokine can be selected from the group consisting of interferon and granulocyte-macrophage colony-stimulating factor.

It is contemplated to be within the scope of the present invention for the heterologous nucleic acid delivered to the stage X primordial germ cells to be an expression vector such as, but not limited to, a viral vector, a plasmid vector, a linear nucleic acid vector or a combination thereof.

Transcriptional promoters of an expression vector of the present invention can be a constitutively active promoter, such as the early/intermediate cytomegalovirus ("CMV") promoter, rous sarcoma virus ("RSV") promoter, or a tissue-specific promoter, preferably a tissue-specific promoter operable in oviduct cells of an avian species including, but not limited to, the promoters of the genes encoding ovalbumin, lysozyme, ovomucoid,

ovotransferrin (conalbumin), and ovomucin. Optionally, the transcriptional promoter of an expression vector can be a regulatable promoter.

The transcriptional terminator of an expression vector can further comprise a region encoding a transcriptional terminator, such as a bovine growth hormone transcriptional
5 terminator.

The present invention provides a transfected avian embryonic stage X primordial germ cell, wherein the stage X primordial germ cell is isolated from an avian embryo according to the method of the present invention, and wherein the stage X primordial germ cell is transfected with a heterologous nucleic acid encoding a heterologous protein desired
10 to be expressed by a transgenic avian.

The present invention further provides a transgenic avian producing a heterologous polypeptide in an avian egg, wherein the transgenic avian is produced by the methods of the present invention for transfecting isolated avian stage X primordial germ cells and delivering the transfected stage X primordial germ cells to a recipient avian embryo for
15 development into a mature avian, wherein the mature avian contains at least one heterologous nucleic acid sequence encoding the polypeptide and wherein the polypeptide is delivered to the white of an avian egg by a female of the avian. In one embodiment of this aspect of the present invention, the transgenic avian contains a transcription unit comprising a heterologous nucleotide sequence encoding a desired polypeptide, a transcription
20 promoter, and a transcriptional terminator operatively linked to the nucleotide sequence encoding the polypeptide.

The present invention also provides for a heterologous nucleic acid encoding the protein of interest that is embedded in all or a significant portion of the genomic locus. The heterologous nucleic acid downstream of an internal ribosome entry site (IRES) directing its
25 independent translation can be embedded in genes that are expressed in oviduct cells (or other cells resulting in deposition of the gene product in the serum or, preferably egg white) of an avian species such as, but not limited to, ovalbumin, lysozyme, ovomucoid, ovotransferrin (conalbumin), and ovomucin. The heterologous nucleic acid can be inserted into an intron, a 5' untranslated region, or a 3' untranslated region of the gene into which it is
30 embedded. The heterologous nucleic acid can even replace the coding region of the gene into which it is embedded.

Additional objects and aspects of the present invention will become more apparent upon review of the detailed description set forth below when taken in conjunction with the accompanying figures, which are briefly described as follows. Each example is provided by
35 way of explanation of the invention, not as limitation of the invention. In fact, it will be

apparent to those skilled in the art that various modifications, combinations, additions, deletions, and variations can be made in the present invention without departing from the scope or spirit of the invention. For instance, features illustrated or described as part of one embodiment can be used in another embodiment to yield a still further embodiment. It is
5 intended that the present invention covers such modifications, combinations, additions, deletions and variations as come within the scope of the appended claims and their equivalents.

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

10

3.1. Terminology

The term "animal" is used herein to include all vertebrate animals, including humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages.

15 The term "avian" as used herein is intended to refer to any species, subspecies or race of organism of the taxonomic class *ava*, such as, but not limited to, such organisms as chicken, turkey, duck, goose, quail, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and cassowary. The term includes the various known strains of *Gallus gallus*, or chickens, (for example, White Leghorn, Brown Leghorn, Barred-Rock,
20 Sussex, New Hampshire, Rhode Island, Ausstralorp, Minorca, Amrox, California Gray, Italian Partidge-colored), as well as strains of turkeys, pheasants, quails, duck, ostriches and other poultry commonly bred.

The term "nucleic acid" as used herein refers to any natural and synthetic linear and sequential arrays of nucleotides and nucleosides, for example cDNA, genomic DNA,
25 mRNA, tRNA, oligonucleotides, oligonucleosides and derivatives thereof. Representative examples of the nucleic acids of the present invention include bacterial plasmid vectors including expression, cloning, cosmid and transformation vectors such as, but not limited to, pBR322, animal viral vectors such as, but not limited to, modified adenovirus, influenza virus, polio virus, pox virus, retrovirus, and the like, vectors derived from bacteriophage
30 nucleic acid, *e.g.*, plasmids and cosmids, artificial chromosomes, such as but not limited to, Yeast Artificial Chromosomes (YACs) and Bacterial Artificial Chromosomes (BACs), and synthetic oligonucleotides like chemically synthesized DNA or RNA. The term "nucleic acid" further includes modified or derivatised nucleotides and nucleosides such as, but not limited to, halogenated nucleotides such as, but not only, 5-bromouracil, and derivatised
35 nucleotides such as biotin-labeled nucleotides.

The term "isolated nucleic acid" as used herein refers to a nucleic acid that has been removed from other components of the cell containing the nucleic acid or from other components of chemical/synthetic reaction used to generate the nucleic acid. In specific embodiments, the nucleic acid is 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% pure.

5 The techniques used to isolate and characterize the nucleic acids and proteins of the present invention are well known to those of skill in the art and standard molecular biology and biochemical manuals may be consulted to select suitable protocols without undue experimentation. See, for example, Sambrook *et al.*, 2001, *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Press; the content of which is herein
10 incorporated by reference in its entirety.

The terms "transcription regulatory sequences" and "gene expression control regions" as used herein refer to nucleotide sequences that are associated with a gene nucleic acid sequence and which regulate the transcriptional expression of the gene. Exemplary transcription regulatory sequences include enhancer elements, hormone response elements,
15 steroid response elements, negative regulatory elements, and the like. The "transcription regulatory sequences" may be isolated and incorporated into a vector nucleic acid to enable regulated transcription in appropriate cells of portions of the vector DNA. The "transcription regulatory sequence" may precede, but is not limited to, the region of a nucleic acid sequence that is in the region 5' of the end of a protein coding sequence that
20 may be transcribed into mRNA. Transcriptional regulatory sequences may also be located within a protein coding region, in regions of a gene that are identified as "intron" regions, or may be in regions of nucleic acid sequence that are in the region of nucleic acid.

The term "promoter" as used herein refers to the DNA sequence that determines the site of transcription initiation by an RNA polymerase. A "promoter-proximal element" may
25 be a regulatory sequence within about 200 base pairs of the transcription start site. A "magnum-specific" promoter, as used herein, is a promoter that is primarily or exclusively active in the tubular gland cells of the avian magnum. Useful promoters also include exogenously inducible promoters. These are promoters that can be "turned on" in response to an exogenously supplied agent or stimulus, which is generally not an endogenous
30 metabolite or cytokine. Examples include an antibiotic-inducible promoter, such as a tetracycline-inducible promoter, a heat-inducible promoter, a light-inducible promoter, or a laser inducible promoter (*e.g.*, Halloran *et al.*, 2000, *Development* 127: 1953-1960; Gerner *et al.*, 2000, *Int. J. Hyperthermia* 16:171-81; Rang and Will, 2000, *Nucleic Acids Res.* 28: 1120-5; Hagihara *et al.*, 1999, *Cell Transplant.* 8: 4314; Huang *et al.*, 1999, *Mol. Med.* 5: 129-37; Forster *et al.*, 1999, *Nucleic Acids Res.* 27: 708-10; Liu *et al.*, 1998, *Biotechniques*
35

24: 624-8, 630-2, the contents of which have been incorporated herein by reference in their entireties).

5 The term "coding region" as used herein refers to a continuous linear arrangement of nucleotides that can be translated into a protein. A full length coding region is translated into a full length protein; that is, a complete protein as would be translated in its natural state absent any post-translational modifications. A full length coding region can also include any leader protein sequence or any other region of the protein that can be excised naturally from the translated protein.

10 The terms "operably" or "operatively linked" refer to the configuration of the coding and control sequences so as to perform the desired function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence and regulating in which tissues, at what developmental timepoints, or in response to which signals, etc., a gene is expressed. A coding sequence is operably linked to or under the control of transcriptional regulatory regions in a cell when DNA polymerase will bind
15 the promoter sequence and transcribe the coding sequence into mRNA that can be translated into the encoded protein. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences, can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered
20 "operably linked" to the coding sequence. Such intervening sequences include but are not limited to enhancer sequences which are not transcribed or are not bound by polymerase.

The term "expressed" or "expression" as used herein refers to the transcription from a gene to give an RNA nucleic acid molecule complementary at least in part to a region of one of the two nucleic acid strands of the gene. The term "expressed" or "expression" as
25 used herein also refers to the translation from said RNA nucleic acid molecule to give a protein or polypeptide or a portion thereof.

The term "nucleic acid vector" as used herein refers to a natural or synthetic single or double stranded plasmid or viral nucleic acid molecule, or any other nucleic acid molecule, such as but not limited to viral vectors and non-viral vectors such as YACs,
30 BACs, bacteriophage-derived artificial chromosome (BBPAC), cosmid or P1 derived artificial chromosome (PAC), that can be transfected or transformed into cells and replicate independently of, or within, the host cell genome. A circular double stranded vector can be linearized by treatment with an appropriate restriction enzyme based on the nucleotide sequence of the vector. A nucleic acid can be inserted into a vector by cutting the vector

35

with restriction enzymes and ligating the pieces together. The nucleic acid molecule can be RNA or DNA.

The term "expression vector" as used herein refers to a nucleic acid vector that comprises regulatory sequences operably linked to a nucleotide sequence coding at least one polypeptide. As used herein, the term "regulatory sequences" includes promoters, enhancers, and other elements that may control gene expression.

The term "transfecting agent" as used herein refers to a composition of matter added to the genetic material for enhancing the uptake of heterologous DNA segment(s) into a eukaryotic cell, preferably an avian cell, and more preferably an avian male germ cell. The enhancement is measured relative to the uptake in the absence of the transfecting agent. Examples of transfecting agents include adenovirus-transferrin-polylysine-DNA complexes. These complexes generally augment the uptake of DNA into the cell and reduce its breakdown during its passage through the cytoplasm to the nucleus of the cell. These complexes can be targeted to the male germ cells using specific ligands that are recognized by receptors on the cell surface of the germ cell, such as the c-kit ligand or modifications thereof. Other preferred transfecting agents include but are not limited to lipofectin, lipofectamine, DIMRIE C, Supeffect, and Effectin (Qiagen), unifactin, maxifactin, DOTMA, DOGS (Transfectam; dioctadecylamidoglycylspermine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOTAP (1,2-dioleoyl-3-trimethylammonium propane), DDAB (dimethyl dioctadecylammonium bromide), DHDEAB (N,N-di-n-hexadecyl-N,N-dihydroxyethyl ammonium bromide), HDEAB (N-n-hexadecylN,N-dihydroxyethylammonium bromide), polybrene, or poly(ethylenimine) (PEI). These non-viral agents have the advantage that they can facilitate stable integration of heterologous DNA sequences into the vertebrate genome, without size restrictions commonly associated with virus-derived transfecting agents.

The terms "transformation" and "transfection" as used herein refer to the process of inserting a nucleic acid into a host. Many techniques are well known to those skilled in the art to facilitate transformation or transfection of a nucleic acid into a prokaryotic or eukaryotic organism. These methods involve a variety of techniques, such as treating the cells with high concentrations of salt such as, but not only a calcium or magnesium salt, an electric field, detergent, or liposome mediated transfection, to render the host cell competent for the uptake of the nucleic acid molecules, and by such methods as sperm-mediated and restriction-mediated integration.

The term "recombinant cell" refers to a cell that has a new combination of nucleic acid segments that are not covalently linked to each other in nature in that particular

configuration. A new configuration of nucleic acid segments can be introduced into an organism using a wide array of nucleic acid manipulation techniques available to those skilled in the art. A recombinant cell can be a single eukaryotic cell, such as a mammalian cell, or a single prokaryotic cell. The recombinant cell may harbor a vector that is
5 extragenomic. An extragenomic nucleic acid vector does not insert into the cell's genome. A recombinant cell may further harbor a vector or a portion thereof (*e.g.*, the portion containing the regulatory sequences and the coding sequence) that is intragenomic. The term intragenomic defines a nucleic acid construct incorporated within the recombinant cell's genome.

10 The terms "recombinant nucleic acid" and "recombinant DNA" as used herein refer to a combination of at least two nucleic acids that is not naturally found in a eukaryotic or prokaryotic cell in that particular configuration. The nucleic acids may include, but are not limited to, nucleic acid vectors, gene expression regulatory elements, origins of replication, suitable gene sequences that when expressed confer antibiotic resistance, protein-encoding
15 sequences and the like. The term "recombinant polypeptide" is meant to include a polypeptide produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location, purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that normally observed in nature.

20 As used herein, the term "transgene" means a nucleic acid sequence (encoding, for example, a human interferon polypeptide) that is partly or entirely heterologous, *i.e.*, exogenous or foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in
25 such a way as to alter the genome of the cell into which it is inserted (*e.g.*, it is inserted at a location that differs from that of the natural gene or its insertion results in a knockout). A transgene also includes a regulatory sequence designed to be inserted into the genome such that it regulates the expression of an endogenous coding sequence, *e.g.*, to increase expression and or to change the timing and or tissue specificity of expression, etc. (*e.g.*, to
30 effect "gene activation").

As used herein, a "transgenic avian" is any avian species, including the chicken, in which one or more of the cells of the avian may contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques known in the art, and particularly, as described herein. The nucleic acid is introduced into a cell, directly
35 or indirectly by introduction into a precursor of the cell, by way of deliberate genetic

manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization (although it does include fertilization with sperm into which a transgene has been introduced, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic avian, the transgene causes cells to express a recombinant form of the subject polypeptide, *e.g.* either agonistic or antagonistic forms, or a form in which the gene has been disrupted. The terms "chimeric avian" or "mosaic avian" are used herein to refer to avians in which the recombinant gene is found, or in which the recombinant is expressed in at least one, preferably some (*e.g.*, 0.1%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%) of the germ cells, but not all cells of the avian. The term "tissue-specific chimeric avian" indicates that the recombinant gene is present and/or expressed in some tissues but not others.

The term "antibody" as used herein refers to polyclonal and monoclonal antibodies and fragments thereof, and immunologic binding equivalents thereof. The term "antibody" refers to a homogeneous molecular entity, or a mixture such as a polyclonal serum product made up of a plurality of different molecular entities, and may further comprise any modified or derivatised variant thereof that retains the ability to specifically bind an epitope. A monoclonal antibody is capable of selectively binding to a target antigen or epitope. Antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, camelized single chain antibodies (scFvs), Fab fragments, F(ab')₂ fragments, disulfide-linked Fvs (sdFv) fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, intrabodies, synthetic antibodies, and epitope-binding fragments of any of the above.

The term "immunoglobulin polypeptide" as used herein refers to a polypeptide derived from a constituent polypeptide of an immunoglobulin. An "immunoglobulin polypeptide" may be, but is not limited to, an immunoglobulin (preferably an antibody) heavy or light chain and may include a variable region, a diversity region, joining region and a constant region or any combination, variant or truncated form thereof. The term "immunoglobulin polypeptides" further includes single-chain antibodies comprised of, but not limited to, an immunoglobulin heavy chain variable region, an immunoglobulin light chain variable region and optionally a peptide linker.

The term "male germ cells" as used herein refers to spermatozoa (*i.e.*, male gametes) and developmental precursors thereof. In fetal development, primordial germ cells are thought to arise from the embryonic ectoderm, and are first seen in the epithelium of the

endodermal yolk sac at the E8 stage. From there they migrate through the hindgut endoderm to the genital ridges. In the sexually mature male vertebrate animal, there are several types of cells that are precursors of spermatozoa, and which can be genetically modified, including the primitive spermatogonial stem cells, known as AO/As, which
5 differentiate into type B spermatogonia. The latter further differentiate to form primary spermatocytes, and enter a prolonged meiotic prophase during which homologous chromosomes pair and recombine. Useful precursor cells at several morphological/developmental stages are also distinguishable: preleptotene spermatocytes, leptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, secondary,
10 spermatocytes, and the haploid spermatids. The latter undergo further morphological changes during spermatogenesis, including the reshaping of their nucleus, the formation of aerosome, and assembly of the tail. The final changes in the spermatozoon (i.e., male gamete) take place in the genital tract of the female, prior to fertilization.

The term "primordial germ cells" as used herein refers to embryonic stem cells that
15 develop into the germ cells of the late stage embryo and hence into the gamete-producing cells of the sexually mature adult animal. Primordial germ cells of an avian appear at stages VII – IX of embryo development. At stage X they begin to develop specific surface markers SSEA-1 and EMA-1. At stages XI-XIV the cells translocate from the ventral surface of the area pellucida to the dorsal hypoblast.

20 The term "stromal cell" as used herein refers to those cells of a tissue that are not primordial germ cells or germ cells.

The term "substantially free" as used herein refers to a population of cells wherein the majority of cells are stage X primordial germ cells. Substantially free does not imply that there are no other non-primordial cells present, just that a percentage of cells in the
25 population are stage X primordial germ cells, for example, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the cells in a population are stage X primordial germ cells.

The term "isolated tissue" as used herein refers to a tissue such as a blastoderm that has been removed from its natural environment, such as an avian egg. The term "isolated
30 cells" as used herein can refer to a population of cells such as stage X primordial germ cells substantially free of other cell types including those stromal cells that naturally coexist with primordial germ cells in a stage X avian embryo.

The term "enriched" as used herein refers to a population of primordial germ cells that constitutes a significantly higher fraction primordial germ cells than non-primordial
35 germ (stromal) cells. Enriched does not imply that there are no other non-primordial cells

present, just that the relative amount of primordial germ cells has been significantly increased, for example, by 1 fold, 2 fold, 5 fold, 10 fold, 50 fold, 100 fold, 500 fold, 1000 fold, 10,000 fold, 100,000 fold, or 1,000,000 fold.

5

3.2. Abbreviations

Abbreviations used in the present specification include the following: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; nt, nucleotide(s); SSC, sodium chloride-sodium citrate; DMSO, dimethyl sulfoxide; MAR; matrix attachment region; TPLSM, two photon laser scanning microscopy; REMI, restriction enzyme mediated
10 integration; DMEM, Dulbecco's Modified Eagles Medium; PGC, primordial germ cell.

4. DESCRIPTION OF DRAWINGS

FIGS. 1A-1B. Periodic Acid Schiff (PAS) staining of PGC and control cells. FIG. 1A shows the negative uptake of stain when sedimented stage X blastodermal
15 stromal cells are stained with PAS. FIG. 1B shows stage X primordial germ cells (PGCs), isolated from a chicken blastoderm, staining positive for PAS.
FIG. 2. Stage X primordial germ cells (PGCs), isolated from a chicken blastoderm, staining for alkaline phosphatase.

20

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for isolating avian stage X primordial germ cells that can be genetically modified and then microinjected into the blastoderm of a recipient egg. It is further contemplated that isolated primordial germ cells can be transfected *ex vivo* so that the chimeric bird can have a population of germ cells that
25 comprise heterologous nucleic acid therein. The resultant transgenic avian can be used for subsequent breeding to generate heterozygous or homozygous transgenic birds.

One aspect of the present invention, are methods for isolating a population of avian stage X primordial germ cells, comprising the steps of obtaining an avian embryo having a stage X blastoderm, isolating the stage X blastoderm from the avian embryo, releasing or
30 dissociating a population of cells from the isolated stage X blastoderm, wherein the population of cells includes stage X primordial germ cells, incubating the released population of cells in a culture medium, for a time sufficient for the stromal cells in the population to sediment from the culture medium, and isolating from the culture medium (*i.e.*, supernatant) a population of cells enriched in stage X primordial germ cells. In one
35 embodiment of the method of the present invention, the population of cells is released or

dissociated from an isolated stage X blastoderm by proteolytic digestion of the blastoderm.

In the various embodiments of the methods of the present invention, the avian egg can be obtained from, for example, any of the group consisting of chicken, turkey, quail, pheasant, duck, goose and ratite. In one embodiment of the method of the present invention, the avian egg is a chicken egg.

In one embodiment of the methods of the present invention, the stage X primordial germ cells have alkaline phosphatase activity and are positive for Periodic Acid Schiff (PAS) staining.

Another aspect of the present invention is methods for generating a transgenic avian having a heterologous germ cell therein, comprising the steps of obtaining an avian egg having a stage X blastoderm, isolating the stage X blastoderm from the avian egg, releasing a population of cells from the isolated stage X blastoderm, wherein the population of cells includes embryonic stage X primordial germ cells therein, incubating the released population of cells in a culture medium, whereby stromal cells sediment from the culture medium, isolating from the culture medium a population of cells enriched in stage X primordial germ cells, microinjecting the isolated population of enriched primordial germ cells into a recipient embryo of an avian egg, and allowing the recipient embryo to develop and hatch as a chick having a heterologous germ cell therein.

One embodiment of the method for generating an avian having a heterologous germ cell therein further comprises the step of allowing the chick having the heterologous germ cell to develop to an adult bird. The adult bird can be a chimera, wherein at least some (preferably 20%, 30%, 50%, 60%, 70%, 80%, 90%) of the germ cells thereof are heterologous germ cells.

In the embodiments of the methods of the present invention for generating an avian having a heterologous germ cell therein, the avian can be selected from the group consisting of chicken, turkey, quail, pheasant, duck, goose and ratite. In one embodiment, the avian is a chicken.

Yet another aspect of the present invention are methods for the production of transgenic avians capable of producing a heterologous polypeptide, comprising the steps of obtaining an avian egg having a stage X blastoderm, isolating the stage X blastoderm from the avian egg, releasing a population of cells from the isolated stage X blastoderm, wherein the population of cells includes stage X primordial germ cells therein, incubating the released population of cells in a culture medium, whereby stromal cells sediment from the culture medium, isolating from the culture medium a population of cells enriched (e.g., by 1 fold, 2 fold, 5 fold, 10 fold, 50 fold, 100 fold, 500 fold, 1000 fold, 10,000 fold,

100,000 fold, or 1,000,000 fold) in stage X primordial germ cells, generating a transfected avian stage X primordial germ cell by delivering a heterologous nucleic acid to the population of cells enriched in stage X primordial germ cells, wherein the heterologous nucleic acid comprises an expression cassette, microinjecting the transfected avian stage X
5 primordial germ cell into a recipient embryo of an avian egg, allowing the recipient embryo to hatch as a chick and mature as an adult bird having a heterologous transfected germ cell therein. Breeding the adult bird having a heterologous transfected germ cell therein with a second adult bird can produce a first transgenic progeny bird heterozygous for the heterologous nucleic acid. Subsequently, mating the heterozygous first transgenic progeny
10 bird with a heterozygous second transgenic progeny bird can generate a homozygous or heterozygous transgenic progeny bird depending upon the genotype of the second bird. In the embodiments of the methods of the present invention, the avian can be selected from the group consisting of chicken, turkey, quail, pheasant, duck, goose and ratite. In one embodiment, the avian is a chicken.

15 In one embodiment of the methods of the present invention for the production of transgenic avians capable of producing a heterologous polypeptide, a heterologous polypeptide encoded by the heterologous nucleic acid can be produced by any of the group consisting of the first heterozygous transgenic bird, the second heterozygous transgenic bird and homozygous transgenic bird, or any progeny thereof.

20 In one embodiment of the methods of the present invention for the production of transgenic avians capable of producing a heterologous polypeptide, a heterologous polypeptide is in the serum of the transgenic bird. In another embodiment of the methods of the present invention for the production of transgenic avians capable of producing a heterologous polypeptide, the heterologous polypeptide is delivered to the white of a
25 developing avian egg produced by the transgenic bird.

In still another embodiment of the present invention the expression cassette comprises a promoter, a transcription termination sequence and a polypeptide-encoding sequence.

In yet another embodiment of the methods of the present invention for the
30 production of transgenic avians capable of producing a heterologous polypeptide, the expression cassette comprises a transcription unit encoding a first heterologous polypeptide, and optionally a second heterologous polypeptide, operably linked to a avian specific transcription promoter, a transcription terminator, and optionally an internal ribosome entry site (IRES).

35

In another embodiment of the methods of the present invention for the production of transgenic avians capable of producing a heterologous polypeptide, the transgenic avian expresses a first and a second transgene encoding a first and a second heterologous polypeptide, and wherein the method further comprises the step of combining the first and
5 second heterologous polypeptides, thereby forming a multimeric protein.

In yet another embodiment, the heterologous nucleic acid encoding the protein of interest that is embedded in all or a significant portion of the genomic locus. The heterologous nucleic acid downstream of an internal ribosome entry site (IRES) directing its independent translation can be embedded in genes found in oviduct cells of an avian species
10 such as, but not limited to, ovalbumin, lysozyme, ovomucoid, ovotransferrin (conalbumin), and ovomucin. The heterologous nucleic acid can be inserted into an intron, a 5' untranslated region, or a 3' untranslated region of the gene of which it is embedded. The heterologous nucleic acid can even replace the coding region of the gene of which it is embedded.

15 In one embodiment of the methods of the present invention, the heterologous polypeptide is selected from the group consisting of a cytokine, hormone, enzyme, structural protein, and immunoglobulin.

In other embodiments of the methods of the present invention, the cytokine can be selected from the group consisting of interferon, interleukin, granulocyte colony-stimulating
20 factor; granulocyte-macrophage colony-stimulating factor; stem cell factor, erythropoietin, thrombopoietin and stem cell factor.

In still other embodiments of the methods of the present invention, the cytokine can be selected from the group consisting of interferon, granulocyte-macrophage colony-stimulating factor, and erythropoietin.

25 In another embodiment of the methods of the present invention, the hormone is selected from the group consisting of insulin, insulin-like growth factor, growth hormone, and human growth hormone.

It is contemplated to be within the scope of the present invention for the heterologous nucleic acid delivered to the stage X primordial germ cells to be an expression
30 vector such as, but not limited to, a viral vector, a plasmid vector, or a linear nucleic acid vector or a combination thereof. The expression vector can be any suitable viral vector, for example, avian leucosis virus, adenoviral vectors, transferrin-polylysine enhanced adenoviral vectors, human immunodeficiency virus vectors, lentiviral vectors, Moloney murine leukemia virus-derived vectors and the like, and virus-derived DNAs that facilitate
35 polynucleotide uptake by and release into the cytoplasm of germ cells.

Transcriptional promoters of an expression cassette or vector of the present invention can be a constitutively active promoter such as the early/intermediate cytomegalovirus promoter, or a tissue-specific promoter, preferably a tissue-specific promoter operable in oviduct cells of an avian species including, but not limited to, the
5 promoters of the genes encoding ovalbumin, lysozyme, ovomucoid, ovotransferrin (conalbumin), and ovomucin. Optionally, the transcriptional promoter of an expression vector can be a regulatable promoter.

The transcriptional terminator of at least one expression vector can further comprises a region encoding a transcriptional terminator, such as a bovine growth hormone
10 transcriptional terminator.

Another aspect of the present invention is a transgenic avian producing a heterologous polypeptide in an avian egg, wherein the transgenic avian is produced by the methods of the present invention for transfecting an isolated avian stage X germ cell and delivering the transfected stage X primordial germ cell to a recipient avian embryo for
15 development into a mature avian, wherein the mature avian comprises at least one heterologous nucleic acid sequence encoding the polypeptide and wherein the polypeptide is delivered to the white of an avian egg.

In one embodiment of this aspect of the present invention, the transgenic avian comprises a transcription unit comprising a heterologous nucleotide sequence encoding at
20 least one polypeptide, a transcription promoter and a transcriptional terminator operatively linked to the nucleotide sequence encoding at least one polypeptide.

Another aspect of the present invention is a transfected avian embryonic stage X primordial germ cell, wherein the stage X primordial germ cell is isolated from an avian egg according to the method of the present invention, and wherein the stage X primordial germ
25 cell is transfected with a heterologous nucleic acid encoding a heterologous protein desired to be expressed by a transgenic avian. The transfected avian primordial germ cell can be delivered to an avian embryo whereupon the transfected germ cells can become incorporated into the cell mass of the embryo. Subsequent development of the egg will allow the transfected germ cells to give rise to a population of germ cells in a mature bird.
30 Breeding of this bird can result in the generation of transgenic progeny birds capable of expressing the heterologous nucleic acid and thereby delivering a desired heterologous polypeptide to a specific tissue or in the serum of the bird.

Yet another aspect of the present invention is a transgenic avian producing a protein, preferably an antibody, in an avian serum, wherein the transgenic avian comprises at least
35 one heterologous nucleic acid sequence encoding one chain of the antibody, and wherein an

antibody is delivered to the serum of the avian egg white.

In one embodiment of this aspect of the present invention, the transgenic avian comprises a transcription unit comprising a heterologous nucleotide sequence encoding at least one polypeptide, a transcription promoter and a transcriptional terminator operatively
5 linked to the nucleotide sequence encoding at least one polypeptide.

5.1. Isolation of Avian Stage X Primordial Germ Cells

The methods of the present invention are methods for isolating a population of cells enriched for primordial germ cells from stage X avian blastoderms. In these methods, the
10 blastoderm of a stage X embryo comprising stromal and primordial germ cells is isolated by dissection from a freshly laid egg and disrupted to yield viable individual or loosely clumped blastodermal cells that include a subpopulation of primordial germ cells. The dissected blastoderm can be disrupted by any method that does not negatively impact the viability of the cells released from the tissue. Suitable methods include, but are not limited
15 to, proteolytic digestion using, for example, trypsin, chymotrypsin, elastase, collagenase or the like and combinations thereof. The blastoderm can be treated with a chelating agent such as, for example, ethylenediaminetetracetate, sodium salt (EDTA) or the like or a combination of the protease and chelating agent. It is further contemplated, however, that any other tissue disruptive method can be employed, including mechanical methods, that
20 will release individual stromal cells and PGCs into a culture medium or buffer and will not significantly decrease the viability or proliferative capacity of the cells. A single blastoderm can give about 5×10^4 cells that are suspended in a culture medium such as, for example, DMEM, or any culture medium that supports blastoderm cell culture. The cell suspension can be placed in a suitably-sized culture plate well. An optional coverslip can be placed in
25 the bottom of the well to collect sedimented cells for microscopic examination.

Incubation of the cell suspension for about 24 hours allows stromal non-primordial germ cells to sediment to the bottom of the culture plate well. The primordial germ cells (PGCs), which are now substantially free of stromal cells, *i.e.*, at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, or even 100% of primordial germ cells, remain in suspension in the
30 medium, thereby comprising a population enriched in stage X primordial germ cells.

The cell suspension can then be centrifuged or filtered to concentrate the enriched stage X PGC population. A typical yield of stage X PGCs from a single blastoderm is between about 200 and about 300 PGCs. Stage X PGCs can be identified and the degree of isolation from other blastodermal cells determined by a positive Periodic Acid Schiff (PAS)
35 staining reaction, and the presence of alkaline phosphatase activity. Stromal cells are

negative for both tests.

Stage X primordial germ cells isolated by the methods of the present invention are suitable target cells for *ex vivo* transfection to generate transgenic primordial germ cells having a heterologous nucleic acid therein. Transfected avian embryonic primordial germ cells can be reintroduced by, for example, microinjection into or below the blastodermal layer of a viable stage X avian embryo and then incubated to produce a transgenic chicken (or other avian species) that will carry the transgene in the its germ-line tissue.

5.2. Transfection of Stage X Primordial Germ Cells

5.2.1. Recombinant Nucleic Acids

The recombinant DNA nucleic acid molecules of the present invention can be expressed in eukaryotic cells by recombinant expression vectors designed for the expression of the encoded proteins. Useful vectors can comprise constitutive or inducible promoters to direct expression of either fusion or non-fusion proteins. With fusion vectors, a number of amino acids are usually added to the expressed target gene sequence such as, but not limited to, a protein sequence for thioredoxin. A proteolytic cleavage site can further be introduced at a site between the target recombinant protein and the fusion sequence. Additionally, a region of amino acids such as a polymeric histidine region can be introduced to allow binding of the fusion protein to metallic ions, such as nickel, bonded to a solid support, and thereby allow purification of the fusion protein. Once the fusion protein has been purified, the cleavage site allows the target recombinant protein to be separated from the fusion sequence. Enzymes suitable for use in cleaving the proteolytic cleavage site include, but are not limited to, Factor Xa and thrombin. Fusion expression vectors that can be useful in the present invention include pGex (Amrad Corp., Melbourne, Australia), pRIT5 (Pharmacia, Piscataway, NJ) and pMAL (New England Biolabs, Beverly, MA), that fuse glutathione S-transferase, protein A, or maltose E binding protein, respectively, to the target recombinant protein.

Expression of a foreign gene can be obtained using eukaryotic host cells such as avian cells. The use of eukaryotic host cells permit partial or complete post-translational modification such as, but not only, glycosylation and/or the formation of the relevant inter- or intra-chain disulfide bonds. Examples of vectors useful for expression in the chicken *Gallus gallus* include pYepSecl as in Baldari *et al.*, 1987, E.M.B.O.J., 6: 229-234 and pYES2 (Invitrogen Corp., San Diego, CA), incorporated herein by reference in their entireties.

The recombinant DNA nucleic acid molecules of the present invention can be delivered to cells using conventional recombinant DNA technology. The recombinant DNA molecules can be inserted into a cell to which the recombinant DNA molecule is heterologous (*i.e.*, not normally present). Alternatively, as described more fully below, the recombinant DNA molecule can be introduced into cells which normally contain the recombinant DNA molecule, for example, to correct a deficiency in the expression of a polypeptide, or where over-expression of the polypeptide is desired.

For expression in heterologous systems, the heterologous DNA molecule is inserted into an expression system or vector of the present invention in proper sense orientation and correct reading frame. The vector can contain the necessary elements for the transcription and translation of the inserted protein-coding sequences such as, for example, an isolated lysozyme gene expression control region, an ovalbumin promoter, an artificial promoter construct and the like.

U.S. Patent No. 4,237,224 to Cohen and Boyer, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), and "Current Protocols in Molecular Biology," Ausubel *et al.*, eds., Greene Publishing Associates (1989) which are hereby incorporated by reference in their entireties, describe the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced to a cell by means of transformation and replicated in cultures, including eukaryotic cells grown in tissue culture.

One aspect of the present invention, therefore, is an expression vector suitable for delivery to a recipient cell for expression of the vector therein. It is contemplated to be within the scope of the present invention for the expression vector to comprise any suitable avian tissue-specific or tissue-restricted promoter, such as an avian lysozyme gene expression control region, an ovalbumin promoter, an ovomucoid promoter, an artificial promoter construct and the like, operably linked to a nucleic acid insert encoding a polypeptide, and optionally a polyadenylation signal sequence. The expression vector of the present invention can further comprise a bacterial plasmid sequence, a viral nucleic acid sequence, or fragments or variants thereof that can allow for replication of the vector in a suitable host.

Nucleic acid sequences or derivative or truncated variants thereof, can be introduced into viruses, such as a vaccinia virus. Methods for making a viral recombinant vector useful for expressing a protein under the control of the lysozyme promoter are analogous to the methods disclosed in U.S. Patent Nos. 4,603,112; 4,769,330; 5,174,993; 5,505,941;

5,338,683; 5,494,807; 4,722,848; Paoletti, E. Proc. Natl. Acad. Sci. 93: 11349-11353 (1996); Moss, B., Proc. Natl. Acad. Sci. 93: 11341-11348 (1996); Roizman, Proc. Natl. Acad. Sci. 93: 11307-11302 (1996); Frolov *et al.*, Proc. Natl. Acad. Sci. 93: 11371-11377 (1996); Grunhaus *et al.* Seminars in Virology 3: 237-252 (1993) and U.S. Patent Nos. 5,591,639; 5,589,466; and 5,580,859 relating to DNA expression vectors, *inter alia*, the contents of which are incorporated herein by reference in their entireties.

Recombinant viruses can also be generated by transfection of plasmids into cells infected with virus. In particular, useful vectors include, bacteriophages such as lambda derivatives, such as λ gt11, λ gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV40, pBLUESCRIPT® II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from STRATAGENE®, La Jolla, Calif., which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see Studier, F.W. *et al.*, 1990, "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes" *Gene Expression Technology* 185, which is hereby incorporated by reference) and any derivatives thereof, cosmid vectors and, in preferred embodiments, artificial chromosomes, such as, but not limited to, Yeast Artificial Chromosomes ("YACs"), Bacterial Artificial Chromosomes ("BACs"), bacteriophage-derived artificial chromosomes ("BBPACs") or P1 derived artificial chromosomes ("PACs"). Such artificial chromosomes are useful in that a large nucleic acid insert can be propagated and introduced into the avian cell. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook *et al.*, Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), hereby incorporated by reference in its entirety.

A variety of host-vector systems can be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. The use of eukaryotic recipient host cells permits partial or complete post-translational modification such as, but not only, glycosylation and/or the formation of the relevant inter- or intra-chain disulfide bonds. Host-vector systems include, but are not limited to, the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; vertebrate cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus) or avian embryonic cells inoculated with the recombinant nucleic acid. The expression elements of these vectors vary in their strength and specificities. Depending

upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Once an avian tissue-specific promoter and a nucleic acid encoding a heterologous protein of the present invention have been cloned into a vector system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. It is contemplated that the incorporation of the DNA of the present invention into a recipient cell can be by any suitable method such as, but not limited to, liposomal transfer, viral transfer, electroporation, gene gun insertion, microinjection and the like. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, and the like. In particular, the present invention contemplates the use of recipient avian stage X primordial germ cells derived from such birds as the chicken or quail. Other hosts can be used, for example, to propagate the construct and intermediates thereof that are ultimately introduced into primordial germ cells.

It is contemplated that the transfected cell according to the present invention can be transiently transfected, whereby the transfected recombinant DNA or expression vector can not be integrated into the genomic nucleic acid. It is further contemplated that the transfected recombinant DNA or expression vector can be stably integrated into the genomic DNA of the recipient cell, thereby replicating with the cell so that each daughter cell receives a copy of the transfected nucleic acid. It is still further contemplated for the scope of the present invention to include a transgenic animal producing a heterologous protein expressed from a transfected nucleic acid according to the present invention.

In one embodiment of the present invention, the transgenic animal is an avian selected from a turkey, duck, goose, quail, pheasant, ratite, and ornamental bird or a feral bird. In another embodiment, the avian is a chicken and the heterologous polypeptide produced under the transcriptional control of the avian promoter according to the present invention is produced in the white of an egg. In yet another embodiment of the present invention, the heterologous polypeptide is produced in the serum of a bird.

5.2.2. Promoters

The vectors of the invention contain promoters that function in avian cells, preferably, that are tissue-specific and, in preferred embodiments, direct expression in the magnum or serum or other tissue such that expressed proteins are deposited in eggs, more preferably, that are specific for expression in the magnum. Alternatively, the promoter directs expression of the protein in the serum of the transgenic avian. Introduction of the

vectors of the invention, preferably, generate transgenics that express the heterologous protein in tubular gland cells where it is secreted into the oviduct lumen and deposited, e.g., into the white of an egg. In preferred embodiments, the promoter directs a level of expression of the heterologous protein in the egg white of eggs laid by G₀ and/or G₁ chicks and/or their progeny that is greater than 5 µg, 10 µg, 50 µg, 100 µg, 250 µg, 500 µg, or 750 µg, more preferably greater than 1 mg, 2 mg, 5 mg, 10 mg, 20 mg, 50 mg, 100 mg, 200 mg, 500 mg, 700 mg, 1 gram, 2 grams, 3 grams, 4 grams or 5 grams. Such levels of expression can be obtained using the promoters of the invention.

In preferred embodiments, the promoters of the invention are derived from genes that express proteins present in significant levels in the egg white and/or the serum. For example, the promoter comprises regions of an ovomucoid, ovalbumin, conalbumin, lysozyme or ovotransferrin promoter or any other promoter that directs expression of a gene in an avian, particularly in a specific tissue of interest, such as the magnum or in the serum. Alternatively, the promoter used in the expression vector may be derived from that of the lysozyme gene that is expressed in both the oviduct and macrophages. Portions of two or more of these, and other promoters that function in avians, may be combined to produce effective synthetic promoter.

The promoter may optionally be a segment of the ovalbumin promoter region that is sufficiently large to direct expression of the coding sequence in the tubular gland cells. Other exemplary promoters include the promoter regions of the ovalbumin, lysozyme, ovomucoid, ovotransferrin or ovomucin genes (for example, but not limited to, as disclosed in co-pending United States Patent Application Nos. 09/922,549, filed August 3, 2001 and 10/114,739, filed April 1, 2002, both entitled "Avian Lysozyme Promoter", by Rapp; United States Patent Application No. 09/998,716, filed November 30, 2001, entitled "Ovomucoid Promoter and Methods of Use," by Harvey *et al.*; and U.S. Patent Application No. To Be Assigned, filed September 18, 2002, titled "Production of a Transgenic Avian by Cytoplasmic Injection" by Rapp and Christmas, Attorney Docket No. 11106-006, all of which are incorporated by reference herein in their entireties). Alternatively, the promoter may be a promoter that is largely, but not entirely, specific to the magnum, such as the lysozyme promoter. Other suitable promoters may be artificial constructs such as a combination of nucleic acid regions derived from at least two avian gene promoters.

The ovalbumin gene encodes a 45 kD protein that is also specifically expressed in the tubular gland cells of the magnum of the oviduct (Beato, 1989, Cell 56:335-344). Ovalbumin is the most abundant egg white protein, comprising over 50 percent of the total protein produced by the tubular gland cells, or about 4 grams of protein per large Grade A

egg (Gilbert, "Egg albumen and its formation" in Physiology and Biochemistry of the Domestic Fowl, Bell and Freeman, eds., Academic Press, London, New York, pp. 1291-1329). The ovalbumin gene and over 20 kb of each flanking region have been cloned and analyzed (Lai *et al.*, 1978, Proc. Natl. Acad. Sci. USA 75:2205-2209; Gannon *et al.*, 1979, Nature 278:428-424; Roop *et al.*, 1980, Cell 19:63-68; and Royal *et al.*, 1975, Nature 279:125-132).

The ovalbumin gene responds to steroid hormones such as estrogen, glucocorticoids, and progesterone, which induce the accumulation of about 70,000 ovalbumin mRNA transcripts per tubular gland cell in immature chicks and 100,000 ovalbumin mRNA transcripts per tubular gland cell in the mature laying hen (Palmiter, 1973, J. Biol. Chem. 248:8260-8270; Palmiter, 1975, Cell 4:189-197). The 5' flanking region contains four DNase I-hypersensitive sites centered at -0.25, -0.8, -3.2, and -6.0 kb from the transcription start site. These sites are called HS-I, -II, -III, and -IV, respectively. Promoters of the invention may contain one, all, or a combination of HS-I, HS-II, HS-III and HS-IV.

Hypersensitivity of HS-II and -III are estrogen-induced, supporting a role for these regions in hormone-induction of ovalbumin gene expression.

HS-I and HS-II are both required for steroid induction of ovalbumin gene transcription, and a 1.4 kb portion of the 5' region that includes these elements is sufficient to drive steroid-dependent ovalbumin expression in explanted tubular gland cells (Sanders and McKnight, 1988, Biochemistry 27: 6550-6557). HS-I is termed the negative-response element ("NRE") because it contains several negative regulatory elements which repress ovalbumin expression in the absence of hormone (Haekers *et al.*, 1995, Mol. Endo. 9:1113-1126). Protein factors bind these elements, including some factors only found in oviduct nuclei suggesting a role in tissue-specific expression. HS-II is termed the steroid-dependent response element ("SDRE") because it is required to promote steroid induction of transcription. It binds a protein or protein complex known as Chirp-I. Chirp-I is induced by estrogen and turns over rapidly in the presence of cyclohexamide (Dean *et al.*, 1996, Mol. Cell. Biol. 16:2015-2024). Experiments using an explanted tubular gland cell culture system defined an additional set of factors that bind SDRE in a steroid-dependent manner, including a NF κ B-like factor (Nordstrom *et al.*, 1993, J. Biol. Chem. 268:13193-13202; Schweers and Sanders, 1991, J. Biol. Chem. 266: 10490-10497).

Less is known about the function of HS-III and HS-IV. HS-III contains a functional estrogen response element, and confers estrogen inducibility to either the ovalbumin proximal promoter or a heterologous promoter when co-transfected into HeLa cells with an estrogen receptor cDNA. These data imply that HS-III may play a functional role in the

overall regulation of the ovalbumin gene. Little is known about the function of HS-IV, except that it does not contain a functional estrogen-response element (Kato *et al.*, 1992, Cell 68: 731-742).

In an alternative embodiment of the invention, transgenes containing constitutive
5 promoters are used, but the transgenes are engineered so that expression of the transgene effectively becomes magnum-specific. Thus, a method for producing an exogenous protein in an avian oviduct provided by the present invention involves generating a transgenic avian having two transgenes in its tubular gland cells. One transgene comprises a first coding sequence operably linked to a constitutive promoter. The second transgene comprises a
10 second coding sequence that is operably linked to a magnum-specific promoter, where expression of the first coding sequence is either directly or indirectly dependent upon the cellular presence of the protein expressed by the second coding sequence.

Additional promoters useful in the present invention include inducible promoters, such as the tet operator and the metallothionein promoter which can be induced by
15 treatment with tetracycline and zinc ions, respectively (Gossen *et al.*, 1992, Proc. Natl. Acad. Sci. 89: 5547-5551 and Walden *et al.*, 1987, Gene 61: 317-327; incorporated herein by reference in their entireties).

5.2.2.1. Chicken lysozyme gene expression control region nucleic acid sequences

20 The chicken lysozyme gene is highly expressed in the myeloid lineage of hematopoietic cells, and in the tubular glands of the mature hen oviduct (Hauser *et al.*, 1981, Hematol. and Blood Transfusion 26: 175-178; Schutz *et al.*, 1978, Cold Spring Harbor Symp. Quart. Biol. 42: 617-624) and is therefore a suitable candidate for an efficient promoter for heterologous protein production in transgenic animals. The regulatory region
25 of the lysozyme locus extends over at least 12 kb of DNA 5' upstream of the transcription start site, and comprises a number of elements that have been individually isolated and characterized. The known elements include three enhancer sequences at about -6.1 kb, -3.9 kb, and -2.7 kb (Grewal *et al.*, 1992, Mol. Cell Biol. 12: 2339-2350; Bonifer *et al.*, 1996, J. Mol. Med. 74: 663-671), a hormone responsive element (Hecht *et al.*, 1988, E.M.B.O.J. 7:
30 2063-2073), a silencer element and a complex proximal promoter. The constituent elements of the lysozyme gene expression control region are identifiable as DNAase 1 hypersensitive chromatin sites (DHS). They may be differentially exposed to nuclease digestion depending upon the differentiation stage of the cell. For example, in the multipotent progenitor stage of myelomonocytic cell development, or in erythroblasts, the
35 silencer element is a DHS. At the myeloblast stage, a transcription enhancer located -6.1

kb upstream from the gene transcription start site is a DHS, while at the later monocytic stage another enhancer, at -2.7 kb becomes DNAase sensitive (Huber *et al.*, 1995, DNA and Cell Biol. 14: 397-402).

This invention also envisions the use of promoters other than the lysozyme promoter, including but not limited to, a cytomegalovirus promoter, an ovomucoid, conalbumin or ovotransferrin promoter or any other promoter that directs expression of a gene in an avian, particularly in a specific tissue of interest, such as the magnum.

Another aspect of the methods of the present invention is the use of combinational promoters comprising an artificial nucleic acid construct having at least two regions wherein the regions are derived from at least two gene promoters, including but not limited to a lysozyme, ovomucoid, conalbumin or ovotransferrin promoter. In one embodiment of the present invention, the promoter may comprise a region of an avian ovomucoid promoter and a region of an avian oxotransferrin promoter, thereby generating the MDOT avian artificial promoter construct as described in U.S. Patent Application No. To Be Assigned, filed September 18, 2002, titled "Production of a Transgenic Avian by Cytoplasmic Injection" by Rapp and Christmas, Attorney Docket No. 11106-006, the disclosure of which is incorporated by reference in its entirety. The MDOT promoter is useful for allowing expression of a heterologous protein in chicken oviduct cells and may be operably linked to any nucleic acid encoding a heterologous polypeptide of interest including, for example, a cytokine, growth hormone, growth factor, enzyme, structural protein or the like.

5.2.3. Codon-Optimized Gene Expression

Another aspect of the present invention is to provide nucleic acid sequences encoding heterologous polypeptides that are codon-optimized for expression in avian cells, and derivatives and fragments thereof.

When a recombinant DNA is to be delivered to a recipient cell for expression therein, the sequence of the nucleic acid sequence can be modified so that the codons are optimized for the codon usage of the recipient species. For example, if the recombinant DNA is transfected into a recipient chicken cell, the sequence of the expressed nucleic acid insert is optimized for chicken codon usage. This can be determined from the codon usage of at least one, and preferably more than one, protein expressed in a chicken cell. For example, the codon usage can be determined from the nucleic acid sequences encoding the proteins ovalbumin, lysozyme, ovomucin and ovotransferrin of chicken as described in Example 8 below.

In one exemplary embodiment of the recombinant DNA of the present invention, a nucleic acid insert encodes the human interferon $\alpha 2b$ polypeptide optimized for codon-usage by the chicken. The nucleic acid sequence and origin of the avian codon-optimized human $\alpha 2b$ is fully disclosed in U.S. Patent Application Serial No. 09/173,864 and PCT Application No. 99/19472, incorporated herein by reference in their entireties. Codon optimization of the sequence is useful in elevating the level of translation in avian eggs.

It is contemplated to be within the scope of the present invention for any nucleic acid encoding a polypeptide to be optimized for expression in avian cells. It is further contemplated that the codon usage can be optimized for a particular avian species used as a source of the host cells. In one embodiment of the present invention, the heterologous polypeptide is encoded using codons optimized for a chicken.

5.2.4. Recombinant Expression Vectors

A variety of vectors useful in carrying out the methods of the present invention are described herein. The introduction of a vector nucleic acid into the isolated stage X PGCs can be performed with embryonic PGCs that are either freshly isolated or in culture. The transgenic cells are then typically injected into the subgerminal cavity beneath a recipient blastoderm in an egg. These vectors can be used for stable introduction of a heterologous coding sequence into the genome of an avian PGC. Suitable vectors can be used that allow expression of exogenous proteins in specific tissues of an avian, and in the oviduct in particular. Vectors can also be selected that allow a hen to produce eggs that contain a heterologous protein, particularly in the white of the egg.

Vectors especially useful for transfecting and generating random, stable integration into the avian genome can contain a coding sequence and a magnum-specific promoter in operational and positional relationship to express the coding sequence in the tubular gland cell of the magnum of the avian oviduct. For instance, the promoter can be derived from the promoter regions of the ovalbumin, lysozyme, conalbumin, ovomucoid, or ovomucin genes. Alternatively, the promoter can be a promoter that is largely, but not entirely, specific to the magnum, such as the lysozyme promoter.

In particular embodiments, useful vectors include, bacteriophages such as lambda derivatives, such as λ gt11, λ gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV40, PBLUESCRIPT® II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from STRATAGENE®, La Jolla, Calif., which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see Studier, F.W. *et. al.*, 1990,

"Use of T7 RNA Polymerase to Direct Expression of Cloned Genes" Gene Expression Technology 185, which is hereby incorporated by reference) and any derivatives thereof, cosmid vectors and, in preferred embodiments, artificial chromosomes, such as, but not limited to, YACs, BACs, BBPACs or PACs. Such artificial chromosomes are useful in that
5 a large nucleic acid insert can be propagated and introduced into the avian cell.

In other particular embodiments, the vectors of the invention are derived from eukaryotic viruses, preferably avian viruses, and can be replication competent or, preferably, replication deficient. In particular embodiments, the vectors are derived from REV, ALV or MuLV. Nucleic acid sequences or derivative or truncated variants thereof, may be
10 introduced into viruses such as vaccinia virus. Methods for making a viral recombinant vector useful for expressing a protein under the control of the lysozyme promoter are analogous to the methods disclosed in U.S. Patent Nos. 4,603,112; 4,769,330; 5,174,993; 5,505,941; 5,338,683; 5,494,807; 4,722,848; Paoletti, E., 1996, Proc. Natl. Acad. Sci. 93: 11349-11353; Moss, 1996, Proc. Natl. Acad. Sci. 93: 11341-11348; Roizman, 1996, Proc.
15 Natl. Acad. Sci. 93: 11307-11302; Frolov *et al.*, 1996, Proc. Natl. Acad. Sci. 93: 11371-11377; Grunhaus *et al.*, 1993, Seminars in Virology 3: 237-252 and U.S. Patent Nos. 5,591,639; 5,589,466; and 5,580,859 relating to DNA expression vectors, *inter alia*; the contents of which are incorporated herein by reference in their entireties.

Recombinant viruses can also be generated by transfection of plasmids into cells
20 infected with virus.

Preferably, vectors can replicate (*i.e.*, have a bacterial origin of replication) and be manipulated in bacteria (or yeast) and can then be introduced into avian cells. Preferably, the vector comprises a marker that is selectable and/or detectable in bacteria or yeast cells and, preferably, also in avian cells, such markers include, but are not limited to, Amp^r, tet^r,
25 LacZ, etc. Preferably, such vectors can accommodate (*i.e.*, can be used to introduce into cells and replicate) large pieces of DNA such as genomic sequences, for example, large pieces of DNA consisting of at least 25 kb, 50 kb, 75 kb, 100 kb, 150 kb, 200 kb or 250 kb, such as BACs, YACs, cosmids, etc.

The insertion of a DNA fragment into a vector can, for example, be accomplished by
30 ligating the DNA fragment into a vector that has complementary cohesive termini.

However, if the complementary restriction sites used to fragment the DNA are not present in the vector, the ends of the DNA molecules may be enzymatically modified.

Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized

oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and the transgene may be modified by homopolymeric tailing.

The vector can be cloned using methods known in the art, *e.g.*, by the methods disclosed in Sambrook *et al.*, 2001, *Molecular Cloning, A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, N.Y.; Ausubel *et al.*, 1989, *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y., both of which are hereby incorporated by reference in their entireties. Preferably, the vectors contain cloning sites, for example, restriction enzyme sites that are unique in the sequence of the vector and insertion of a sequence at that site would not disrupt an essential vector function, such as replication.

As discussed above, vectors used in certain methods of the invention preferably can accommodate, and in certain embodiments comprise, large pieces of heterologous DNA such as genomic sequences, particularly avian genomic sequences. Such vectors can contain an entire genomic locus, or at least sufficient sequence to confer endogenous regulatory expression pattern, *e.g.*, high level of expression in the magnum characteristic of lysozyme, ovalbumin, ovomucoid, ovotransferrin, etc, and to insulate the expression of the transgene sequences from the effect of regulatory sequences surrounding the site of integration of the transgene in the genome. Accordingly, as detailed below, in preferred embodiments, the transgene is inserted in an entire genomic loci or significant portion thereof.

To manipulate large genomic sequences contained in, for example, a BAC, nucleotide sequences coding for the heterologous protein to be expressed and/or other regulatory elements may be inserted into the BAC by directed homologous recombination in bacteria, *e.g.*, the methods of Heintz WO 98/59060; Heintz *et al.*, WO 01/05962; Yang *et al.*, 1997, *Nature Biotechnol.* 15: 859-865; Yang *et al.*, 1999, *Nature Genetics* 22: 327-35; which are incorporated herein by reference in their entireties.

Alternatively, the BAC can also be engineered or modified by "E-T cloning," as described by Muyrers *et al.* (1999, *Nucleic Acids Res.* 27(6): 1555-57, incorporated herein by reference in its entirety). Using these methods, specific DNA may be engineered into a BAC independently of the presence of suitable restriction sites. This method is based on homologous recombination mediated by the *recE* and *recT* proteins ("ET-cloning") (Zhang *et al.*, 1998, *Nat. Genet.* 20(2): 123-28; incorporated herein by reference in its entirety). Homologous recombination can be performed between a PCR fragment flanked by short homology arms and an endogenous intact recipient such as a BAC. Using this method, homologous recombination is not limited by the disposition of restriction endonuclease

cleavage sites or the size of the target DNA. A BAC can be modified in its host strain using a plasmid, *e.g.*, pBAD- $\alpha\beta\gamma$, in which *recE* and *recT* have been replaced by their respective functional counterparts of phage lambda (Muyrers *et al.*, 1999, *Nucleic Acids Res.* 27(6): 1555-57). Preferably, a BAC is modified by recombination with a PCR product containing
5 homology arms ranging from 27-60 bp. In a specific embodiment, homology arms are 50 bp in length.

In another embodiment, a transgene is inserted into a yeast artificial chromosome (YAC) (Burke *et al.*, 1987, *Science* 236: 806-12; and Peterson *et al.*, 1997, *Trends Genet.* 13:61, both of which are incorporated by reference herein in their entireties).

10 In other embodiments, the transgene is inserted into another vector developed for the cloning of large segments of genomic DNA, such as a cosmid or bacteriophage P1 (Sternberg *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87: 103-07). The approximate maximum insert size is 30-35 kb for cosmids and 100 kb for bacteriophage P1. In another embodiment, the transgene is inserted into a P-1 derived artificial chromosome (PAC)
15 (Mejia *et al.*, 1997, *Genome Res* 7:179-186). The maximum insert size is 300 kb.

Vectors containing the appropriate heterologous sequences may be identified by any method well known in the art, for example, by sequencing, restriction mapping, hybridization, PCR amplification, *etc.*

The vectors of the invention comprise one or more nucleotide sequences encoding a
20 heterologous protein desired to be expressed in the transgenic avian, as well as regulatory elements such as promoters, enhancers, MARs, IRES's and other translation control elements, transcriptional termination elements, polyadenylation sequences, *etc.* In particular embodiments, the vector of the invention contains at least two nucleotide sequences coding for heterologous proteins, for example, but not limited to, the heavy and light chains of an
25 immunoglobulin.

In a preferred embodiment, the nucleotide sequence encoding the heterologous protein is inserted into all or a significant portion of a nucleic acid containing the genomic sequence of an endogenous avian gene, preferably an avian gene that is expressed in the magnum, *e.g.*, lysozyme, ovalbumin, ovomucoid, conalbumin, ovotransferrin, *etc.* For
30 example, the heterologous gene sequence may be inserted into or replace a portion of the 3' untranslated region (UTR) or 5' untranslated region (UTR) or an intron sequence of the endogenous gene genomic sequence. Preferably, the heterologous gene coding sequence has its own IRES. For descriptions of IRESes, *see, e.g.*, Jackson *et al.*, 1990, *Trends Biochem Sci.* 15(12):477-83; Jang *et al.*, 1988, *J. Virol.* 62(8):2636-43; Jang *et al.*, 1990,
35 *Enzyme* 44(1-4):292-309; and Martinez-Salas, 1999, *Curr. Opin. Biotechnol.* 10(5):458-64;

Palmenberg *et al.*, United States Patent No. 4,937,190, which are incorporated by reference herein in their entireties. In another embodiment, the heterologous protein coding sequence is inserted at the 3' end of the endogenous gene coding sequence. In another preferred embodiment, the heterologous gene coding sequences are inserted using 5' direct fusion
5 wherein the heterologous gene coding sequences are inserted in-frame adjacent to the initial ATG sequence (or adjacent the nucleotide sequence encoding the first two, three, four, five, six, seven or eight amino acids) of the endogenous gene or replacing some or all of the sequence of the endogenous gene coding sequence. In yet another specific embodiment, the heterologous gene coding sequence is inserted into a separate cistron in the 5' region of the
10 endogenous gene genomic sequence and has an independent IRES sequence.

The present invention further relates to nucleic acid vectors and transgenes inserted therein that incorporate multiple polypeptide-encoding regions, wherein a first polypeptide-encoding region is operatively linked to a transcription promoter and a second polypeptide-encoding region is operatively linked to an IRES. For example, the vector may contain
15 coding sequences for two different heterologous proteins (*e.g.*, the heavy and light chains of an immunoglobulin) or the coding sequences for all or a significant part of the genomic sequence for the gene from which the promoter driving expression of the transgene is derived, and the heterologous protein desired to be expressed (*e.g.*, a construct containing the genomic coding sequences, including introns, of the avian lysozyme gene when the
20 avian lysozyme promoter is used to drive expression of the transgene, an IRES, and the coding sequence for the heterologous protein desired to be expressed downstream (*i.e.*, 3' on the RNA transcript of the IRES)). Thus, in certain embodiments, the nucleic acid encoding the heterologous protein is introduced into the 5' untranslated or 3' untranslated regions of an endogenous gene, such as but not limited to, lysozyme, ovalbumin, ovotransferrin, and
25 ovomucoid, with an IRES sequence directing translation of the heterologous sequence.

Such nucleic acid constructs, when inserted into the genome of a bird and expressed therein, will generate individual polypeptides that may be post-translationally modified, for example, glycosylated or, in certain embodiments, form complexes, such as heterodimers with each other in the white of the avian egg. Alternatively, the expressed polypeptides may
30 be isolated from an avian egg and combined *in vitro*, or expressed in a non-reproductive tissue such as serum. In other embodiments, for example, but not limited to, when expression of both heavy and light chains of an antibody is desired, two separate constructs, each containing a coding sequence for one of the heterologous proteins operably linked to a promoter (either the same or different promoters), are introduced by microinjection into
35 cytoplasm of one or more embryonic cells and transgenic avians harboring both transgenes

in their genomes and expressing both heterologous proteins are identified. Alternatively, two transgenic avians each containing one of the two heterologous proteins (*e.g.*, one transgenic avian having a transgene encoding the light chain of an antibody and a second transgenic avian having a transgene encoding the heavy chain of the antibody) can be bred
5 to obtain an avian containing both transgenes in its germline and expressing both transgene encoded proteins, preferably in eggs.

Recombinant expression vectors can be designed for the expression of the encoded proteins in eukaryotic cells. Useful vectors may comprise constitutive or inducible promoters to direct expression of either fusion or non-fusion proteins. With fusion vectors,
10 a number of amino acids are usually added to the expressed target gene sequence such as, but not limited to, a protein sequence for thioredoxin, a polyhistidine, or any other amino acid sequence that facilitates purification of the expressed protein. A proteolytic cleavage site may further be introduced at a site between the target recombinant protein and the fusion sequence. Additionally, a region of amino acids such as a polymeric histidine region
15 may be introduced to allow binding of the fusion protein to metallic ions such as nickel bonded to a solid support, and thereby allow purification of the fusion protein. Once the fusion protein has been purified, the cleavage site allows the target recombinant protein to be separated from the fusion sequence. Enzymes suitable for use in cleaving the proteolytic cleavage site include, but are not limited to, Factor Xa and thrombin. Fusion expression
20 vectors that may be useful in the present invention include pGex (AMRAD® Corp., Melbourne, Australia), pRIT5 (PHARMACIA®, Piscataway, NJ) and pMAL (NEW ENGLAND BIOLABS®, Beverly, MA), fusing glutathione S-transferase, protein A, or maltose E binding protein, respectively, to the target recombinant protein.

Once a promoter and a nucleic acid encoding a heterologous protein of the present
25 invention have been cloned into a vector system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. It is contemplated that the incorporation of the DNA of the present invention into a recipient cell may be by any suitable method such as, but not limited to, viral transfer, electroporation, gene gun insertion, sperm-
30 mediated transfer to an ovum, microinjection and the like. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, and the like. In particular, the present invention contemplates the use of recipient avian cells, such as chicken cells or quail cells.

Another aspect of the present invention, therefore, is a method of expressing a
35 heterologous polypeptide in a eukaryotic cell by transfecting an avian cell with a

recombinant DNA comprising an avian tissue-specific promoter operably linked to a nucleic acid insert encoding a polypeptide and, optionally, a polyadenylation signal sequence, and culturing the transfected cell in a medium suitable for expression of the heterologous polypeptide under the control of the avian lysozyme gene expression control region.

5 Yet another aspect of the present invention is a eukaryotic cell transformed with an expression vector according to the present invention and described above. In one embodiment of the present invention, the transformed cell is a chicken oviduct cell and the nucleic acid insert comprises the chicken lysozyme gene expression control region, a nucleic acid insert encoding a human interferon $\alpha 2b$ and codon optimized for expression in
10 an avian cell, and an SV40 polyadenylation sequence.

In another embodiment, the transformed cell is a quail oviduct cell and the nucleic acid insert comprises the artificial avian promoter construct MDOT operably linked to an interferon-encoding sequence, as described in U.S. Patent Application No. To Be Assigned, filed September 18, 2002, titled "Production of a Transgenic Avian by Cytoplasmic
15 Injection" by Rapp and Christmas, Attorney Docket No. 11106-006, the disclosure of which is incorporated by reference in its entirety.

In yet another embodiment of the present invention, a quail oviduct cell is transfected with the nucleic acid insert comprising the MDOT artificial promoter construct operably linked to an erythropoietin (EPO)-encoding nucleic acid, wherein the transfected
20 quail produces heterologous erythropoietin.

5.2.4.1. Viral Host Cell Transformation

Another suitable approach for *in vivo* introduction of nucleic acid and the associated gene expression control regions into a recipient cell is by use of a viral vector containing
25 nucleic acid, *e.g.* a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, *e.g.*, by a cDNA contained in the viral vector, are expressed efficiently in cells that have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be
30 the recombinant gene delivery system of choice for the transfer of heterologous genes *in vivo*. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. Recombinant retrovirus can be constructed wherein the retroviral coding sequences (gag, pol, env) have been replaced by nucleic acid encoding a polypeptide, thereby rendering the retrovirus replication
35 defective. Protocols for producing recombinant retroviruses and for infecting cells *in vitro*

or *in vivo* with such viruses can be found in Sections 9.10-9.14 of "Current Protocols in Molecular Biology," Ausubel *et al.*, eds., Greene Publishing Associates (1989) and other standard laboratory manuals. Examples of suitable retroviruses well known to those skilled in the art include, but are not limited to, pLJ, pZIP, pWE and pEM. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include, but are not limited to, psiCrip, psiCre, psi2 and psiAm.

Furthermore, it is possible to limit the infection spectrum of retroviruses, and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO 93/25234, WO 94/06920, and WO 94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include coupling antibodies specific for cell surface antigens to the viral env protein (Roux *et al.*, 1989, Proc. Natl. Acad. Sci. 86: 9079-9083; Julan *et al.*, 1992, J. Virol. 73: 3251-3255; and Goud *et al.*, 1983, Virology 163: 251-254), or coupling cell surface ligands to the viral env proteins (Neda *et al.*, 1991, J. Biol. Chem. 266: 14143-14146); the contents of which are incorporated herein by reference in their entireties. Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the env protein to a sialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/env fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector into an amphotropic vector. Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences that control expression of the nucleic acid encoding an immunoglobulin polypeptide of the retroviral vector.

One retrovirus useful for randomly introducing a transgene into the avian genome is the replication-deficient ALV retrovirus. To produce an appropriate ALV retroviral vector, a pNLB vector is modified by inserting a region of the ovalbumin promoter and one or more exogenous genes between the 5' and 3' long terminal repeats (LTRs) of the retrovirus genome. Any coding sequence placed downstream of the ovalbumin promoter will be expressed at high levels and only in the tubular gland cells of the oviduct magnum because the ovalbumin promoter drives the high level of expression of the ovalbumin protein and is only active in the oviduct tubular gland cells. While a 7.4 kb ovalbumin promoter has been found to produce the most active construct when assayed in cultured oviduct tubular gland cells, the ovalbumin promoter must be shortened for use in the retroviral vector. In a preferred embodiment, the retroviral vector comprises a 1.4 kb segment of the ovalbumin promoter; a 0.88 kb segment would also suffice.

Any of the vectors of the present invention can also optionally include a coding sequence encoding a signal peptide that will direct secretion of the protein expressed by the vector's coding sequence from the tubular gland cells of the oviduct. This aspect of the invention effectively broadens the spectrum of exogenous proteins that can be deposited in avian eggs using the methods of the invention. Where an exogenous protein would not otherwise be secreted, the vector bearing the coding sequence is modified to comprise a DNA sequence comprising about 60 bp encoding a signal peptide from the lysozyme gene. The DNA sequence encoding the signal peptide is inserted in the vector such that it is located at the N-terminus of the protein encoded by the cDNA.

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner *et al.*, 1988, *BioTechniques* 6: 616; Rosenfeld *et al.*, 1991, *Science* 252: 431-434; and Rosenfeld *et al.*, 1992, *Cell* 68: 143-155; the contents of which are incorporated herein by reference in their entireties. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 and the like) are well known to those skilled in the art. The virus particle is relatively stable and amenable to purification and concentration and, as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (*e.g.*, retroviral DNA). Most replication-defective adenoviral vectors currently in use, and therefore favored by the present invention, are deleted with respect to parts or all of the viral E1 and E3 genes, but retain as much as 80% of the adenoviral genetic material (see, for example, Jones *et al.*, 1979, *Cell* 16: 683; Berkner *et al.*, *supra*; and Graham *et al.*, pp. 109-127 in "Methods in Molecular Biology," vol. 7, E. J. Murray, ed., Humana Publishing, Clifton, N.J., 1991) (the contents of which are incorporated herein by reference in their entireties). Expression of an inserted nucleic acid encoding a polypeptide including, but not limited to, human interferon $\alpha 2b$, an immunoglobulin, EPO, and GM-CSF can be under the control of, for example, the lysozyme promoter, the ovalbumin promoter, artificial promoter construct sequences and the like.

Yet another viral vector system useful for delivery of the subject nucleic acid encoding, for example, an immunoglobulin polypeptide, is the adeno-associated virus (AAV). Vectors containing as little as 300 base pairs of AAV can be packaged and can

integrate into a host genome, however, space for heterologous DNA is limited to about 4.5 kb. An AAV vector such as that described by Tratschin *et al.* (1985, Mol. Cell. Biol. 5: 3251-3260) can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see, for example, Hermonat *et al.*, 5 1984, Proc. Natl. Acad. Sci. 81: 6466-6470; Tratschin *et al.*, 1985, Mol. Cell. Biol. 4: 2072-2081; Wondisford *et al.*, 1988, Mol. Endocrinol. 2: 32-39; Tratschin *et al.*, 1984, J. Virol. 51: 611-619; and Flotte *et al.*, 1993, J. Biol. Chem. 268: 3781-3790; the contents of which are incorporated herein, incorporated herein by reference in their entirety.

Other viral vector systems that can have application in the methods according to the 10 present invention have been derived from, but are not limited to, herpes virus, vaccinia virus, avian leucosis virus and several RNA viruses.

5.2.4.2. Non-Viral Expression Vectors

Most non-viral methods of gene transfer rely on normal mechanisms used by 15 eukaryotic cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject nucleic acid encoding a polypeptide by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, polylysine conjugates, and artificial viral envelopes.

20 In a representative embodiment of the present invention, a nucleic acid encoding a polypeptide can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (see, *inter alia*, PCT publication WO 91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075), incorporated herein by 25 reference in their entirety.

In similar fashion, the gene delivery system comprises an antibody or cell surface ligand that is cross-linked with a gene binding agent such as polylysine (see, for example, PCT publications WO 93/04701, WO 92/22635, WO 92/20316, WO 92/19749, and WO 92/06180)(the contents of which are incorporated herein by reference in their entirety). It 30 will also be appreciated that effective delivery of the subject nucleic acid constructs via receptor-mediated endocytosis can be improved using agents which enhance escape of gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan *et al.*, 1993, Science 260-926; Wagner 35 *et al.*, 1992, Proc. Natl. Acad. Sci. 89: 7934; and Christiano *et al.*, 1993, Proc. Natl. Acad.

Sci. 90: 2122; incorporated herein by reference in their entireties). It is further contemplated that a recombinant DNA molecule can be delivered to a recipient host cell by other non-viral methods including by gene gun, microinjection, or the like.

5.2.4.3. Matrix Attachment Regions

In preferred embodiments of the invention, the vectors contain matrix attachment regions (MARs) that preferably flank the transgene sequences to reduce position effects on expression when integrated into the avian genome. In fact, 5' MARs and 3' MARs (also referred to as "scaffold attachment regions" or SARs) have been identified in the outer boundaries of the chicken lysozyme locus (Phi-Van *et al.*, 1988, E.M.B.O.J. 7: 655-664; 10 Phi-Van, L. and Stratling, W.H., 1996, Biochem. 35: 10735-10742). Deletion of a 1.32 kb or a 1.45 kb halves region, each comprising half of a 5' MAR, reduces positional variation in the level of transgene expression (Phi-Van and Stratling, *supra*).

The 5' matrix-associated region (5' MAR), located about -11.7 kb upstream of the chicken lysozyme transcription start site, can increase the level of gene expression by limiting the positional effects exerted against a transgene (Phi-Van *et al.*, 1988, *supra*). At least one other MAR is located 3' downstream of the protein encoding region. Although MAR nucleic acid sequences are conserved, little cross-hybridization is seen, indicating significant overall sequence variation. However, MARs of different species can interact with the nucleomatrixes of heterologous species, to the extent that the chicken lysozyme MAR can associate with the plant tobacco nucleomatrix as well as that of the chicken oviduct cells (Mlynarova *et al.*, 1994, Cell 6: 417-426; von Kries *et al.*, 1990, Nucleic Acids Res. 18: 3881-3885).

Gene expression must be considered not only from the perspective of cis-regulatory elements associated with a gene, and their interactions with trans-acting elements, but also with regard to the genetic environment in which they are located. Chromosomal positioning effects (CPEs), therefore, are the variations in levels of transgene expression associated with different locations of the transgene within the recipient genome. An important factor governing CPE upon the level of transgene expression is the chromatin structure around a transgene, and how it cooperates with the cis-regulatory elements. The cis-elements of the lysozyme locus are confined within a single chromatin domain (Bonifer *et al.*, 1996, *supra*; Sippel *et al.*, pgs. 133-147 in Eckstein F. & Lilley D.M.J. (eds), "Nucleic Acids and Molecular Biology", Vol. 3, 1989, Springer.

The lysozyme promoter region of chicken is active when transfected into mouse
35 fibroblast cells and linked to a reporter gene such as the bacterial chloramphenicol

acetyltransferase (CAT) gene. The promoter element is also effective when transiently transfected into chicken promacrophage cells. In each case, however, the presence of a 5' MAR element increased positional independency of the level of transcription (Stief *et al.*, 1989, Nature 341: 343-345; Sippel *et al.*, pgs. 257 – 265 in Houdebine L.M. (ed),

5 "Transgenic Animals: Generation and Use").

The ability to direct the insertion of a transgene into a site in the genome of an animal where the positional effect is limited offers predictability of results during the development of a desired transgenic animal, and increased yields of the expressed product. Sippel and Steif disclose, in U.S. Patent No. 5,731,178, which is incorporated by reference
10 herein in its entirety, methods to increase the expression of genes introduced into eukaryotic cells by flanking a transcription unit with scaffold attachment elements, in particular the 5' MAR isolated from the chicken lysozyme gene. The transcription unit disclosed by Sippel and Steif was an artificial construct that combined only the -6.1 kb enhancer element and the proximal promoter element (base position -579 to +15) from the lysozyme gene. Other
15 promoter associated elements were not included. However, although individual cis-regulatory elements have been isolated and sequenced, together with short regions flanking DNA, the entire nucleic acid sequence comprising the functional 5' upstream region of the lysozyme gene has not been determined in its entirety and therefore not employed as a functional promoter to allow expression of a heterologous transgene.

20 Accordingly, vectors of the invention comprise MARs, preferably both 5' and 3' MARs that flank the transgene, including the heterologous protein coding sequences and the regulatory sequences.

5.2.5. Transgenesis of Stage X Primordial Germ Cells

25 Using the methods of the present invention, transgenes can be introduced into avian stage X primordial germ cells to produce a transgenic chicken or other avian species carrying the transgene in the genetic material of its germ-line tissue. The methods and vectors of the present invention further are useful in generating transgenic avians capable of expressing heterologous genes in the serum of the avian and/or deposited in an avian egg.
30 The cells useful in the present invention are primordial germ cells (PGCs) isolated from an avian embryo at or around stage X of development. The targeted stage X PGCs can be isolated freshly, maintained in culture, or frozen.

A variety of vectors useful in carrying out the methods of the present invention are described herein. These vectors can be used for the stable introduction of an exogenous
35 coding sequence into the genome of a bird. In alternative embodiments, the vectors can be

used to produce heterologous proteins in specific tissues of an avian, and in the oviduct in particular. In still further embodiments, the vectors are used in methods to produce an avian egg containing a heterologous protein.

In one embodiment of the invention, vectors used for transfecting stage X PGCs
5 resulting in random, stable integration of a heterologous coding sequence into the avian genome contain a coding sequence and a magnum-specific promoter in operational and positional relationship to express the coding sequence in the tubular gland cell of the magnum of the avian oviduct. The magnum-specific promoter can optionally be a segment of the ovalbumin promoter region which is sufficiently large to direct expression of the
10 coding sequence in the tubular gland cells. Other exemplary promoters include the promoter regions of the ovalbumin, lysozyme, conalbumin, ovomucoid, or ovomucin genes. Alternatively, the promoter can be a promoter that is largely, but not entirely, specific to the magnum, such as the lysozyme promoter. Other suitable promoters can be artificial constructs such as a combination of nucleic acid regions derived from at least two avian
15 gene promoters.

In an alternative embodiment of the invention, transgenes containing constitutive promoters are used, but the transgenes are engineered so that expression of the transgene effectively becomes magnum-specific. Thus, a method for producing a heterologous protein in an avian oviduct provided by the present invention involves generating a
20 transgenic avian that bears two transgenes in its tubular gland cells. One transgene comprises a first coding sequence operably linked to a constitutive promoter. The second transgene comprises a second coding sequence that is operably linked to a magnum-specific promoter, where expression of the first coding sequence is either directly or indirectly dependent upon the cellular presence of the protein expressed by the second coding
25 sequence.

Optionally, site-specific recombination systems, such as the Cre-loxP or FLP-FRT systems, are utilized to implement the magnum-specific activation of an engineered constitutive promoter. In one embodiment, the first transgene contains an FRT-bounded blocking sequence which blocks expression of the first coding sequence in the absence of
30 FTP, and the second coding sequence encodes FTP. In another embodiment, the first transgene contains a loxP-bounded blocking sequence which blocks expression of the first coding sequence in the absence of the Cre enzyme, and the second coding sequence encodes Cre. The loxP-bounded blocking sequence can be positioned in the 5' untranslated region of the first coding sequence and the loxP-bounded sequence can optionally contain an open
35 reading frame.

For instance, in one embodiment of the invention, magnum-specific expression is conferred on a constitutive transgene, by linking a cytomegalovirus (CMV) promoter to the coding sequence of the protein to be secreted (CDS). The 5' untranslated region (UTR) of the coding sequence contains a loxP-bounded blocking sequence. The loxP-bounded
5 blocking sequence contains two loxP sites, between which is a start codon (ATG) followed by a stop codon, creating a short, nonsense open reading frame (ORF). Note that the loxP sequence contains two start codons in the same orientation. Therefore, to prevent them from interfering with translation of the coding sequence after loxP excision, the loxP sites must be orientated such that the ATGs are in the opposite strand.

10 In the absence of Cre enzyme, the cytomegalovirus promoter drives expression of a small open reading frame (ORF). Ribosomes will initiate at the first ATG, the start codon of the ORF, then terminate without being able to reinitiate translation at the start codon of the coding sequence. To be certain that the coding sequence is not translated, the first ATG is out of frame with the coding sequence's ATG. If the Cre enzyme is expressed in cells
15 containing the CMV-cDNA transgene, the Cre enzyme will recombine the loxP sites, excising the intervening ORF. Translation will begin at the start codon of the coding sequence, resulting in synthesis of the desired protein.

To make this system tissue specific, the Cre enzyme is expressed under the control of a tissue-specific promoter, such as the magnum-specific ovalbumin promoter, in the same
20 cell as the CMV-loxP-coding sequence transgene. Although a truncated ovalbumin promoter can be fairly weak, it is still tissue-specific and will express sufficient amounts of the Cre enzyme to induce efficient excision of the interfering ORF. In fact, low levels of recombinase should allow higher expression of the recombinant protein since it does not compete against coding sequence transcripts for translation machinery.

25 Alternate methods of blocking translation of the coding sequence include inserting a transcription termination signal and/or a splicing signal between the loxP sites. These can be inserted along with the blocking ORF or alone. In another embodiment of the invention, a stop codon can be inserted between the loxP sites in the signal peptide of the coding sequence. Before recombinase is expressed, the peptide terminates before the coding
30 sequence. After recombinase is expressed (under the direction of a tissue specific promoter), the stop codon is excised, allowing translation of the coding sequence. The loxP site and coding sequence are juxtaposed such that they are in frame and the loxP stop codons are out of frame. Since signal peptides are able to accept additional sequence (Brown *et al.*, 1984, Mol. Gen. Genet. 197:351-7), insertion of loxP or other recombinase
35 target sequences (*i.e.* FRT) is unlikely to interfere with secretion of the desired coding

sequence. In one expression vector, the loxP site is present in the signal peptide such that the amino acids encoded by loxP are not present in the mature, secreted protein. Before Cre enzyme is expressed, translation terminates at the stop codon, preventing expression of β -lactamase. After recombinase is expressed (only in magnum cells), the loxP sites
5 recombine and excise the first stop codon. Therefore, β -lactamase is expressed selectively only in magnum cells.

In the aforementioned embodiments, the blocking ORF can be any peptide that is not harmful to chickens. The blocking ORF can also be a gene that is useful for production of the ALV-transduction particles and/or transgenic birds. In one embodiment, the blocking
10 ORF is a marker gene.

For instance, the blocking ORF could be the neomycin resistance gene, which is required for production of transduction particles. Once the transgene is integrated into the chicken genome, the neomycin resistance gene is not required and can be excised.

Alternatively, β -lactamase can be used as the blocking ORF, as it is a useful marker
15 for production of transgenic birds. As an example, the blocking ORF is replaced by β -lactamase and the downstream coding sequence now encodes a secreted biopharmaceutical. β -Lactamase will be expressed in blood and other tissues; it will not be expressed in the magnum after magnum-specific expression of Cre and recombination-mediated excision of β -lactamase, allowing expression of the desired protein.

The Cre and loxP transgenes could be inserted into the chicken genome via
20 mediated transgenesis either simultaneously or separately. Any method of transgenesis that results in stable integration into the chicken genome is suitable including, but not limited to, viral integration. Both the ovalbumin promoter-recombinase and CMV-loxP-CDS transgenes could be placed simultaneously into chickens. However, the efficiencies of
25 transgenesis are low and therefore the efficiency of getting both transgenes into the chicken genome simultaneously is low. In an alternative and preferred method, one flock is produced that carries the magnum-specific promoter/recombinase transgene and a second is produced that carries the CMV-loxP-CDS transgene. The flocks would then be crossed to each other. Hens resulting from this outbreeding will express the coding sequence and only
30 in their magnum.

As mentioned above, the vectors produced according to the methods of the invention can optionally be provided with a 3' UTR containing a polyadenylation site to confer stability to the RNA produced. In a preferred embodiment, the 3' UTR can be that of the exogenous gene, or selected from the group consisting of the ovalbumin, lysozyme, or SV40
35 late region. However, the ovalbumin 3' UTR is not suitable in a PMGI vector that is to be

inserted into the endogenous ovalbumin gene because the addition of ovalbumin sequences to the PMGI vector will interfere with proper targeting.

5.3. Transgenic Birds

5 After the transfected stage X primordial germ cell population is delivered to a recipient blastoderm by microinjection and the recipient egg resealed and allowed to develop and hatch a viable chick. Typically, the hatchling will be a chimera wherein at least some germ cells of the chick will have originated from the injected transfected PGCs. Therefore, with transfected PGCs at least some chick germ cells will also have heterologous
10 nucleic acid. It is anticipated that the heterologous nucleic acid can form stable transfectants, whereby the heterologous nucleic acid is incorporated into the genome of the recipient avian cells. The resulting transgenic avian chick (*i.e.*, the G_0) will carry one or more desired transgene(s) some or all of its cells, preferably in its germ line. These G_0 transgenic avians can be bred using methods well known in the art to generate second
15 generation (*i.e.*, G_1 s) transgenic avians that carry the transgene, *i.e.*, achieve germline transmission of the transgene. In preferred embodiments, the methods of the invention result in germline transmission, *i.e.*, percentage of G_0 s that transmit the transgene to progeny (G_1 s), that is greater than 5%, preferably, greater than 10%, 20%, 30%, 40%, and, most preferably, greater than 50%, 60%, 70%, 80%, 90% or even 100%. In other embodiments,
20 the efficiency of transgenesis (*i.e.*, number of G_0 s containing the transgene) is greater than 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 99%. The transgenic germ cells of the present invention can, therefore, generate sperm cells comprising a heterologous nucleic acid. Subsequent breeding can produce heterozygous or homozygous birds wherein all cells carry at least one copy of the heterologous nucleic acid. For example, the chimeric
25 hatchling can be bred with a bird not having the heterologous nucleic acid, thereby producing at least one heterozygous offspring.

Cross-breeding heterozygous offspring can result in homozygous and heterozygous birds with respect to the heterologous nucleic acid, according to classic Mendelian inheritance. Progeny, and subsequent generations therefrom, of the initial chimeric bird can
30 have the heterologous nucleic acid in all cells including, but not limited to, germ cells. It is contemplated, however, that if the heterologous nucleic acid, when introduced to the enriched stage X PGCs, does not form integrated stable transfectants, that some progeny birds will be chimeric birds having some cells not having the heterologous nucleic acid therein. It is further contemplated for transgenic birds to be bred by natural coitus or by
35 isolating mature sperm from a male bird and artificially inseminating a recipient female,

using techniques well known to those of skill in the art.

The heterologous nucleic acid can encode a heterologous polypeptide desired to be expressed by a mature transgenic bird. For this purpose, the present invention provides heterologous nucleic acids comprising an expression cassette wherein the polypeptide-
5 encoding nucleic acid can be operably linked to a transcription controlling region such as a tissue-specific promoter. Suitable promoters include, but are not limited to, avian oviduct specific promoters, viral promoters, such as the CMV promoter and the like. It is contemplated that the promoter operably linked to the polypeptide encoding nucleic acid will allow for expression of the polypeptide in a heterozygous or homozygous transgenic
10 avian. The desired heterologous protein can be expressed into the serum of the transgenic bird or, for example, in the case of oviduct cells, into the white of a developing egg.

Another aspect of the present invention concerns transgenic birds such as, but not only, chickens and quails that contain at least one transgene and that preferably (though optionally) express the subject nucleic acid encoding a polypeptide in one or more cells in
15 the animal, such as the oviduct cells of the chicken. Suitable methods for the generation of transgenic avians having heterologous DNA incorporated therein are described, for example, in U.S. Patent Application No: 09/173,864 and incorporated herein by reference in its entirety. In embodiments of the present invention, therefore, the expression of the transgene is restricted to specific subsets of cells, tissues or developmental stages utilizing,
20 for example, cis-acting sequences that control expression in the desired pattern. Toward this end, tissue-specific regulatory sequences, or tissue-specific promoters, and conditional regulatory sequences can be used to control expression of the transgene in certain spatial patterns. Moreover, temporal patterns of expression can be provided by, for example, conditional recombination systems or prokaryotic transcriptional regulatory sequences. The
25 inclusion of a 5' MAR region in the novel isolated lysozyme gene expression control region of the present invention can allow the heterologous expression unit to escape the chromosomal positional effect (CPE) and therefore be expressed at a more uniform level in transgenic tissues that received the transgene by a route other than through germ line cells.

Conditional transgenes can be provided using prokaryotic promoter sequences that
30 require prokaryotic proteins to be simultaneously expressed to facilitate expression of the transgene. Operators present in prokaryotic cells have been extensively characterized *in vivo* and *in vitro* and can be readily manipulated to place them in any position upstream from or within a gene by standard techniques. Such operators comprise promoter regions and regions that specifically bind proteins such as activators and repressors. One example is
35 the operator region of the *lexA* gene of *E. coli* to which the LexA polypeptide binds. Other

exemplary prokaryotic regulatory sequences and the corresponding trans-activating prokaryotic proteins are disclosed by Brent and Ptashne in U.S. Patent No. 4,833,080, and incorporated herein by reference in its entirety. Transgenic animals can be created which harbor the subject transgene under transcriptional control of a prokaryotic sequence that is not appreciably activated by eukaryotic proteins. Breeding of this transgenic animal with another animal that is transgenic for the corresponding prokaryotic trans-activator can permit activation of the nucleic acid encoding an immunoglobulin polypeptide. Moreover, expression of the conditional transgenes can be induced by gene therapy-like methods (such as described above) wherein a gene encoding the trans-activating protein, *e.g.*, a recombinase or a prokaryotic protein, is delivered to the tissue and caused to be expressed, such as in a cell-type specific manner.

Additionally, inducible promoters can be employed, such as the tet operator and the metallothionein promoter that can be induced by treatment with tetracycline and zinc ions, respectively (Gossen *et al.*, 1992, Proc. Natl. Acad. Sci. 89: 5547-5551; and Walden *et al.*, 1987, Gene 61: 317-327; incorporated herein by reference in their entireties.

In the case of an avian, a heterologous polypeptide or polypeptides encoded by the transgenic nucleic acid can be secreted into the oviduct lumen of the mature animal and deposited as a constituent component of the egg white into eggs laid by the animal. It is also contemplated to be within the scope of the present invention for the heterologous polypeptides to be produced in the serum of a transgenic avian. A leaky promoter such as the CMV promoter can be operably linked to a transgene, resulting in expression of the transgene in all tissues of the transgenic avian, resulting in production of a heterologous polypeptide in the serum. Transgenic avians produced by the present invention will have the ability to lay eggs that contain one or more desired heterologous protein(s) or variants thereof.

Accordingly, the invention provides transgenic avians produced by methods of the invention. In preferred embodiments, the transgenic avian contains a transgene comprising a heterologous peptide coding sequence operably linked to a promoter and, in certain embodiments, other regulatory elements. In more preferred embodiments, the transgenic avians of the invention produce heterologous proteins, preferably in a tissue specific manner, more preferably such that they are deposited in the serum and, most preferably, such that the heterologous protein is deposited into the egg, particularly in the egg white. In preferred embodiments, the transgenic avians produce eggs containing greater than 5 μ g, 10 μ g, 50 μ g, 100 μ g, 250 μ g, 500 μ g, or 750 μ g, more preferably greater than 1 mg, 2 mg, 5

mg, 10 mg, 20 mg, 50 mg, 100 mg, 200 mg, 500 mg, 700 mg, 1 gram, 2 grams, 3 grams, 4 grams or 5 grams of the heterologous protein.

5.3.1. Restriction Enzyme-Mediated Integration (REMI)

5 The restriction enzyme-mediated integration ("REMI") method for stably integrating heterologous DNA into the genomic DNA of a recipient cell is described by Shemesh *et al.* in PCT Publication No. WO 99/42569, and incorporated herein by reference in its entirety. This REMI method comprises, in part, an adaptation of the REMI technique disclosed by Schiest and Petes (1991, Proc. Nat. Acad. Sci. U.S.A. 88: 7585-7589 (1991) and Kuspa and
10 Loomis (1992, Proc. Nat. Acad. Sci. U.S.A., 89: 8803-8807), both incorporated herein by reference in their entireties.

The REMI method is suitable for introducing heterologous DNA into the genome nucleic acid of an embryonic cell, including the isolated stage X PGCs of the present invention, or somatic cell of an avian.

15 The heterologous nucleic acid to be integrated into, for example, the PGC nuclear DNA is converted to a linear double stranded DNA possessing single-stranded cohesive ends by contacting the heterologous DNA with a type II restriction enzyme that upon scission, generates such ends. The nucleic acid to be cut can be a circular nucleic acid such as in a plasmid or a viral vector or a linear nucleic acid that possesses at least one
20 recognition and cutting site outside of the genes or regulatory regions critical to the desired post-integration function of the nucleic acid, and no recognition and cutting sites within the critical regions.

Alternatively the heterologous DNA to be integrated into the PGC nuclear DNA can be prepared by chemically and/or enzymatically adding cohesive ends to a linear DNA (see,
25 for example, Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual., 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; incorporated herein by reference in its entirety). The added cohesive ends must be able to hybridize to the cohesive ends characteristic of a nucleic acid cleaved by a type II restriction endonuclease.

Alternatively the cohesive ends can be added by combining the methods based on type II
30 restriction enzyme cutting and chemical and/or enzymatic addition.

According to the present invention, a heterologous nucleic acid encoding at least one polypeptide, and the appropriate restriction enzyme can be introduced into PGCs together or sequentially by way of, for example, electroporation, or lipofection. Preferably electroporation can be used, and most preferably lipofection is used. However, the present
35 invention contemplates that any technique capable of transferring heterologous material into

PGCs could be used. It is understood that the heterologous nucleic acid can be integrated into the genome of a recipient cell. It is further understood that the heterologous nucleic acid can not be integrated into the genome of the recipient cell. The combination of REMI as described in the present application, plus a relatively benign method of transferring
5 heterologous material into a cell can result in heterologous nucleic acid being stably integrated into genomic DNA of a high fraction of the treated stage X PGCs.

It is contemplated to be within the scope of the present invention for nucleic acids encoding polypeptides to be derived from any suitable species including, for example, a human, a mouse, a rat, a rabbit, a goat, a sheep, a cow, a horse or a bird. Antibodies can be
10 monoclonal antibodies. It is further within the scope of the present invention for polypeptides to be modified, for example, by exchanging regions within the polypeptides from one animal species for equivalent regions from another species. It is further understood that a polypeptide from one animal species can be combined with a polypeptide from another animal species.

15 5.4. Production of Exogenous Protein

Methods of the invention that can provide for the production of exogenous or heterologous protein in, for example, the avian oviduct and the production of eggs which contain heterologous protein involve providing a suitable vector and introducing the vector
20 into stage X PGCs so that the vector is integrated into the avian genome. A subsequent step involves deriving a mature transgenic avian from the transgenic PGCs produced in the previous steps. Deriving a mature transgenic avian from the PGCs optionally involves transferring the transgenic PGCs to an embryo and allowing that embryo to develop fully, so that the cells become incorporated into the bird as the embryo is allowed to develop.

25 A transgenic avian so produced from transgenic embryonic cells is known as a founder. Such founders may be mosaic for the transgene. In one embodiment, potential transgenic chicks can be screened using a method of DNA extraction combined with a high-throughput method of gene detection as described in Harvey *et al.*, 2002, Poult. Sci. 81(2):202-212 and U.S. Patent No. 6,423,488, the disclosures of which are incorporated by
30 reference in their entireties.

The invention further provides production of heterologous proteins in other tissues of the transgenic avians. Some founders will carry the transgene in the tubular gland cells in the magnum of their oviducts. These birds will express the exogenous protein encoded by the transgene in their oviducts. If the exogenous protein contains the appropriate signal
35 sequences, it will be secreted into the lumen of the oviduct and into the white of an egg. In

one embodiment of the present invention, the exogenous protein is expressed in the egg white of a transgenic chicken as described in Harvey *et al.*, 2002, Nature Biotechnology 19:396-399, the disclosure of which is incorporated by reference in its entirety.

5 A transgenic bird so produced from the transgenic PGCs is known as a germ-line founder. A germ-line founder is a founder that carries the transgene in genetic material of its germ-line tissue. The germ-line founder may also carry the transgene in oviduct magnum tubular gland cells that express the exogenous protein. Therefore, in one embodiment, the transgenic bird can have tubular gland cells expressing the exogenous protein and the offspring of the transgenic bird will also have oviduct magnum tubular
10 gland cells that express the exogenous protein. Alternatively, the offspring express a phenotype determined by expression of the exogenous gene in a specific tissue of the avian.

The methods and transgenic avians of the present invention can be used to express, in large yields and at low cost, a wide range of desired proteins including those used as human and animal pharmaceuticals, diagnostics, and livestock feed additives. Proteins such
15 as growth hormones, cytokines, structural proteins and enzymes including human growth hormone, interferon, and β -casein are examples of proteins that are desirably expressed in the oviduct and deposited in eggs according to the invention. Other possible proteins to be produced include, but are not limited to, albumin, α -1 antitrypsin, antithrombin III, collagen, factors VIII, IX, X (and the like), fibrinogen, hyaluronic acid, insulin, lactoferrin, protein C,
20 erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), tissue-type plasminogen activator (tPA), feed additive enzymes, somatotropin, and chymotrypsin. Immunoglobulins and genetically engineered antibodies, including immunotoxins that bind to surface antigens on human tumor cells and destroy them, can also be expressed for use as pharmaceuticals or
25 diagnostics.

5.4.1. Multimeric Proteins

The invention, in preferred embodiments, provides methods for producing multimeric proteins, preferably immunoglobulins, such as antibodies, and antigen binding
30 fragments thereof. The multimeric protein is formed from combining a first and a second transgene encoding a first and a second heterologous polypeptide expressed by a transgenic avian.

In one embodiment of the present invention, the multimeric protein is an immunoglobulin, wherein the first and second heterologous polypeptides are an
35 immunoglobulin heavy and light chains respectively. Illustrative examples of this and other

aspects and embodiments of the present invention for the production of heterologous multimeric polypeptides in avian cells are fully disclosed in U.S. Patent Application No. 09/877,374, filed June 8, 2001, by Rapp, which is incorporated herein by reference in its entirety. In one embodiment of the present invention, therefore, the multimeric protein is an immunoglobulin wherein the first and second heterologous polypeptides are an immunoglobulin heavy and light chain respectively. Accordingly, the invention provides immunoglobulin and other multimeric proteins that have been produced by transgenic avians of the invention.

In the various embodiments of this aspect of the present invention, an immunoglobulin polypeptide encoded by the transcriptional unit of at least one expression vector may be an immunoglobulin heavy chain polypeptide comprising a variable region or a variant thereof, and may further comprise a D region, a J region, a C region, or a combination thereof. An immunoglobulin polypeptide encoded by the transcriptional unit of an expression vector may also be an immunoglobulin light chain polypeptide comprising a variable region or a variant thereof, and may further comprise a J region and a C region. It is also contemplated to be within the scope of the present invention for the immunoglobulin regions to be derived from the same animal species, or a mixture of species including, but not only, human, mouse, rat, rabbit and chicken. In preferred embodiments, the antibodies are human or humanized.

In other embodiments of the present invention, the immunoglobulin polypeptide encoded by the transcriptional unit of at least one expression vector comprises an immunoglobulin heavy chain variable region, an immunoglobulin light chain variable region, and a linker peptide thereby forming a single-chain antibody capable of selectively binding an antigen.

Another aspect of the present invention provides a method for the production in an avian of an heterologous protein capable of forming an antibody suitable for selectively binding an antigen comprising the step of producing a transgenic avian incorporating at least one transgene, wherein the transgene encodes at least one heterologous polypeptide selected from an immunoglobulin heavy chain variable region, an immunoglobulin heavy chain comprising a variable region and a constant region, an immunoglobulin light chain variable region, an immunoglobulin light chain comprising a variable region and a constant region, and a single-chain antibody comprising two peptide-linked immunoglobulin variable regions. Preferably, the antibody is expressed such that it is deposited in the white of the developing eggs of the avian. The hard shell avian eggs thus produced can be harvested and the heterologous polypeptide capable of forming or which formed an antibody can be

isolated from the harvested egg. It is also understood that the heterologous polypeptides may also be expressed under the transcriptional control of promoters that allow for release of the polypeptides into the serum of the transgenic animal. Exemplary promoters for non-tissue specific production of a heterologous protein are the CMV promoter and the RSV promoter.

5 In one embodiment of this method of the present invention, the transgene comprises a transcription unit encoding a first and a second immunoglobulin polypeptide operatively linked to a transcription promoter, a transcription terminator and, optionally, an internal ribosome entry site (IRES)(*see*, for example, U.S. Patent No. 4,937,190 to Palmenberg *et al.*, the contents of which is incorporated herein by reference in its entirety).

10 In an embodiment of this method of the present invention, the isolated heterologous protein is an antibody capable of selectively binding to an antigen. In this embodiment, the antibody may be generated within the serum of an avian or within the white of the avian egg by combining at least one immunoglobulin heavy chain variable region and at least one immunoglobulin light chain variable region, preferably cross-linked by at least one disulfide bridge. The combination of the two variable regions will generate a binding site capable of binding an antigen using methods for antibody reconstitution that are well known in the art.

It is, however, contemplated to be within the scope of the present invention for immunoglobulin heavy and light chains, or variants or derivatives thereof, to be expressed in separate transgenic avians, and therefore isolated from separate media including serum or eggs, each isolate comprising a single species of immunoglobulin polypeptide. The method may further comprise the step of combining a plurality of isolated heterologous immunoglobulin polypeptides, thereby producing an antibody capable of selectively binding to an antigen. In this embodiment, two individual transgenic avians may be generated wherein one transgenic produces serum or eggs having an immunoglobulin heavy chain variable region, or a polypeptide comprising such, expressed therein. A second transgenic animal, having a second transgene, produces serum or eggs having an immunoglobulin light chain variable region, or a polypeptide comprising such, expressed therein. The polypeptides may be isolated from their respective sera and eggs and combined *in vitro* to generate a binding site capable of binding an antigen.

30 Examples of therapeutic antibodies that can be used in methods of the invention include but are not limited to HERCEPTIN® (Trastuzumab) (Genentech, CA) which is a humanized anti-HER2 monoclonal antibody for the treatment of patients with metastatic breast cancer; REOPRO® (abciximab) (Centocor) which is an anti-glycoprotein IIb/IIIa

- receptor on the platelets for the prevention of clot formation; ZENAPAX® (daclizumab) (Roche Pharmaceuticals, Switzerland) which is an immunosuppressive, humanized anti-CD25 monoclonal antibody for the prevention of acute renal allograft rejection; PANOREX™ which is a murine anti-17-IA cell surface antigen IgG2a antibody (Glaxo Wellcome/Centocor); BEC2 which is a murine anti-idiotypic (GD3 epitope) IgG antibody (ImClone System); IMC-C225 which is a chimeric anti-EGFR IgG antibody (ImClone System); VITAXIN™ which is a humanized anti- α V β 3 integrin antibody (Applied Molecular Evolution/MedImmune); Campath 1H/LDP-03 which is a humanized anti CD52 IgG1 antibody (Leukosite); Smart M195 which is a humanized anti-CD33 IgG antibody (Protein Design Lab/Kanebo); RITUXAN™ which is a chimeric anti-CD20 IgG1 antibody (IDEC Pharm/Genentech, Roche/Zettyaku); LYMPHOCIDE™ which is a humanized anti-CD22 IgG antibody (Immunomedics); ICM3 is a humanized anti-ICAM3 antibody (ICOS Pharm); IDEC-114 is a primatized anti-CD80 antibody (IDEC Pharm/Mitsubishi); ZEVALIN™ is a radiolabelled murine anti-CD20 antibody (IDEC/Schering AG); IDEC-131 is a humanized anti-CD40L antibody (IDEC/Eisai); IDEC-151 is a primatized anti-CD4 antibody (IDEC); IDEC-152 is a primatized anti-CD23 antibody (IDEC/Seikagaku); SMART anti-CD3 is a humanized anti-CD3 IgG (Protein Design Lab); 5G1.1 is a humanized anti-complement factor 5 (C5) antibody (Alexion Pharm); D2E7 is a humanized anti-TNF- α antibody (CAT/BASF); CDP870 is a humanized anti-TNF- α Fab fragment (Celltech); IDEC-151 is a primatized anti-CD4 IgG1 antibody (IDEC Pharm/SmithKline Beecham); MDX-CD4 is a human anti-CD4 IgG antibody (Medarex/Eisai/Genmab); CDP571 is a humanized anti-TNF- α IgG4 antibody (Celltech); LDP-02 is a humanized anti- α 4 β 7 antibody (LeukoSite/Genentech); OrthoClone OKT4A is a humanized anti-CD4 IgG antibody (Ortho Biotech); ANTOVA™ is a humanized anti-CD40L IgG antibody (Biogen); ANTEGREN™ is a humanized anti-VLA-4 IgG antibody (Elan); and CAT-152 is a human anti-TGF- β_2 antibody (Cambridge Ab Tech).

6. EXAMPLE: ISOLATION OF EMBRYONIC STAGE X PRIMORDIAL GERM CELLS

- Cells were collected from the embryonic stage X blastoderms of freshly laid Barred Rock (BRD) birds. Blastoderms were dissected from the freshly laid eggs and the integrity of the dissected tissue was disrupted by tryptic digestion. Cells (about 50-60 x 10³ cells per blastoderm) that were released from the stage X blastoderms were suspended in 1 x DMEM containing 10% FBS and penicillin/streptomycin solution and plated in 6-well plates. A cover slip was placed in each well to collect cells that settled under gravity for subsequent characterization. After 24 hrs in culture, cells in the supernatant were collected by

centrifugation (500 rpm at room temperature for approximately 10 minutes) and rinsed once with 1 x PBS, yielding the desired cell population highly enriched in embryonic stage X primordial germ cells (PGCs). Typically, a single stage X blastoderm yields between about 200 and about 300 primordial germ cells from a population of about 50,000 blastodermal cells. To identify PGCs, and determine the degree of isolation from other blastodermal cells, cell suspensions were characterized by staining with Periodic Acid Schiff (PAS) and alkaline phosphatase reagents. PGCs are positive when stained with PAS and are positive for alkaline phosphatase activity, while stromal cells are negative for both (Ponce de Leon *et al.*, U.S. Patent No. 6,156,569; Pain *et al.*, 1996, Development 122: 2339–2348)

Periodic Acid Schiff (PAS) staining. PGCs in a total volume of 100-200 µl were placed on a slide and then heated over a flame to fix and adhere the cells to the slide. The slides were then processed using the Periodic Acid Schiff (PAS) staining system according to manufacturer's protocol (Sigma Diagnostics, Inc). Cells that had settled down on the cover slips during the isolation of the PGCs were processed in a similar manner as negative controls.

As shown in FIG. 1 and in Table 1, cells collected from the enriched supernatant stained positive for PAS (FIG. 1A), while control cells were negative for the stain (FIG. 1B).

Table 1: PAS Staining of pre-PGCs

number of cells on slide	number of cells positive for PAS	% of cells positive for PAS
32	30	93%

Alkaline Phosphatase (AP) staining. Cells were stained for alkaline phosphatase activity using a Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories) according to the manufacturer's instructions. As shown in FIG 2, the stage X PGCs in the enriched cell population were positive for alkaline phosphatase activity, in contrast to stromal cells used as negative controls.

The results obtained from staining the enriched PGC fraction, obtained from sedimentation of cells from a blastodermal cell population, demonstrate that the PGC fraction is substantially free of other cell types and subtypes of stromal blastodermal cells.

7. EXAMPLE: MICROINJECTION OF STAGE X PRIMORDIAL GERM CELL
POPULATION TO A RECIPIENT BLASTODERM

After the stage X primordial germ cell population is delivered to a recipient
5 blastoderm by microinjection, the recipient egg resealed and allowed to develop and hatch a
viable chick. The method of U.S. Patent No. 6,397,777 to Andacht *et al.*, which is hereby
incorporated by reference in its entirety, describes such a method, which is summarized
below.

The egg contains an embryo at the blastoderm stage. The egg is manipulated by
10 microinjecting the stage X primordial germ cell population through the opening of the
embryo-containing egg. The opening can be into the area around and in close proximity to
the embryo. A first opening is made in the egg shell, and the underlying egg shell
membrane is cut away. After manipulation of the embryo, the opening in the egg shell is
resealed by applying a heat softened composition, such as a hot melt glue. Any air bubbles
15 introduced into the interior of the egg upon cutting of the underlying egg shell membrane
can optionally be removed by expanding the air sac of the egg through a second opening in
the egg shell and forcing undesirable air bubbles out through the first opening by
displacement of the egg white and the yolk. After the egg is manipulated as described
above, it is then incubated to allow development of the embryo. The incubation is
20 maintained until the embryo is hatched from the egg.

8. EXAMPLE: GERM-LINE TESTING OF THE PGCs

To study the potential of the enriched stage X PGCs for germ-line transmission,
cells collected from blastoderms of black embryos (Barred Rock) were injected into white
25 embryos, with the transmission of black feather color used to germ-line heritability. In the
first experiment, 30 recipient White Leghorn stage X embryos were irradiated to a dosage
level of 600 rads and approximately 150 PGC cells, isolated from black Barred Rock (BRD)
birds as described in Example 6, were microinjected into each embryo. Eleven chicks
hatched with one exhibiting 20% feather color.

30 In the second experiment, PGC suspensions were collected as described above and
passed through a 15 μ m filter. Cells were gently washed from the filter and concentrated by
centrifugation at 500 rpm at room temperature. Stage X PGCs, isolated from three BRD
embryos, were pooled and injected into 29 recipient White Leghorn embryos. Nine chicks
hatched, with two of the chicks exhibiting feather chimerism. Both chimeric birds, numbers
35 2660 and 2661, were males suitable for breeding to BRD hens.

The mature birds displaying chimeric feather color distribution were bred to Barred Rock birds. Because the black phenotype is recessive in chickens, the production of black progeny indicates that the parental chimeras contained germ cells of Barred Rock origin. That is, that at least some of the enriched population of stage X primordial germ cells from black donor blastoderms, injected into white embryonic recipients, developed as germ cells and were passed to progeny. The results are summarized as follows:

Table 2: Results of Breeding Chimeric and Barred Rock Birds.

bird #	# of eggs incubated	# hatch	# white feathers	# black feathers	% progeny with black feathers
male # 2660	482	225	224	1	0.4
male # 2661	328	156	147	7	4.4
female # 2657	124	80	76	4	5
female # 2659	130	56	46	10	17.8
female # 2654	132	94	94	0	0

9. EXAMPLE: PRODUCTION OF FULLY TRANSGENIC G₁ CHICKENS

Males are selected for breeding because a single male can give rise to 20 to 30 G₁ offspring per week, as opposed to 6 G₁ offspring per female per week, thereby speeding the expansion of a G₁ transgenic flock. The feed of G₀ males is supplemented with sulfamethazine to accelerate the sexual maturation of male birds, such that they start producing sperm at 10-12 weeks of age, instead of the usual 20-22 weeks. The use of sulfamethazine to decrease time to maturation does not adversely affect the male birds' health or fertility.

Sperm DNA of all males are screened for the presence of the transgene. Sperm are collected and the DNA extracted using Chelex-100. Briefly, 3 μ l of sperm and 200 μ l of 5% Chelex-100 are mixed, followed by the addition of 2 μ l of 10 mg/ml proteinase K and 7 μ l of 2 M DTT. Samples are incubated at 56° C for 30-60 minutes. Samples are boiled for 8 minutes and vortexed vigorously for 10 seconds. After centrifugation at 10 to 15 G for 2-3 minutes, the supernatant is ready for analysis using a PCR technique, Taqman™ analysis, or the like. Using a Taqman™ assay, for example, the extracted DNA is analyzed using a Taqman™ probe and primers complementary to the transgene. It is estimated that 5%, or 4 to 5 chicks, will have the transgene in their sperm DNA.

G₁ offspring will be obtained through breeding germline transgenic males to non-transgenic, White Leghorn females. Hatched chicks are vent-sexed and screened for the

presence of the transgene in their blood DNA using a Taqman™ assay, PCR assay, western blot, or the like. Twenty male and female G₁ transgenics will be obtained, in approximately 3 – 4 weeks. Males will be kept for further breeding and females tested for the presence of the transgene, and expression of the protein coded by that transgene, in the egg white.

5

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the

10 appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

15

20

25

30

35

WHAT IS CLAIMED IS:

1. A method for isolating a population of avian stage X primordial germ cells, said method comprising the steps of:
 - 5 (a) dissociating a population of cells from an isolated stage X blastoderm, wherein the population of cells includes stage X primordial germ cells and stromal cells;
 - (b) incubating the dissociated population of cells in a culture medium for a time sufficient for said stromal cells to sediment from the culture medium; and
 - 10 (c) isolating from the culture medium a population of cells enriched in stage X primordial germ cells.
2. The method of Claim 1, wherein the avian egg is obtained from any of the
15 group consisting of chicken, turkey, quail, pheasant, duck, goose and ratite.
3. The method of Claim 1, wherein the avian egg is a chicken egg.
4. The method of Claim 1, wherein the stage X primordial germ cells have
20 alkaline phosphatase activity.
5. The method of Claim 1, wherein the stage X primordial germ cells are positive for Periodic Acid Schiff staining.
- 25 6. The method of Claim 1, wherein the population of cells is dissociated from the isolated stage X blastoderm by proteolytic digestion of the blastoderm.
7. A method for generating an avian having a heterologous germ cell therein, said method comprising the steps of:
 - 30 (a) dissociating a population of cells from an isolated stage X blastoderm, wherein the population of cells includes stage X primordial germ cells and stromal cells;
 - (b) incubating the dissociated population of cells in a culture medium for a time sufficient for said stromal cells to sediment from the culture medium;
 - 35

- 5
- (c) isolating from the culture medium a population of cells enriched in stage X primordial germ cells;
 - (d) delivering the population of cells isolated in step (c) into a recipient avian embryo; and
 - (e) allowing the recipient embryo to hatch as a chick having a heterologous germ cell therein.

10 8. The method of Claim 7, further comprising the step: allowing the chick having the heterologous germ cell to develop to an adult bird.

9. The method of Claim 7, wherein the chick is a chimera, and wherein at least some of the germ cells of said chick are heterologous germ cells.

15 10. The method of Claim 7, wherein the avian is selected from the group consisting of chicken, turkey, quail, pheasant, duck, goose, and ratite.

11. The method of Claim 7, wherein the avian is a chicken.

20 12. A method for the production of a transfected avian stage X primordial germ cell, said method comprising the steps of:

- (a) dissociating a population of cells from an isolated stage X blastoderm, wherein the population of cells includes stage X primordial germ cells and stromal cells;
 - (b) incubating the dissociated population of cells in a culture medium for a time sufficient for said stromal cells to sediment from the culture medium;
 - (c) isolating from the culture medium a population of cells enriched in stage X primordial germ cells; and
 - (d) transfecting one or more of said avian stage X primordial germ cells in said population with a heterologous nucleic acid.
- 30

13. The method of Claim 12, wherein the heterologous nucleic acid comprises an expression cassette.

35

14. The method of Claim 12, wherein the heterologous nucleic acid comprises a vector.

15. The method of Claim 13, wherein the expression cassette comprises a promoter, a transcription termination sequence and a polypeptide-encoding sequence.

16. The method of Claim 13, wherein the expression cassette comprises a transcription unit encoding a first heterologous polypeptide, and optionally a second heterologous polypeptide, operably linked to a avian specific transcription promoter, a transcription terminator, and optionally an internal ribosome entry site (IRES).

17. The method of Claim 16, further comprising a step of combining the first and second heterologous polypeptides to form a multimeric protein.

18. The method of Claim 17, wherein the multimeric protein is an immunoglobulin.

19. The method of Claim 14, wherein the vector is selected from the group consisting of a viral vector, a plasmid vector and a linear nucleic acid.

20. The method of Claim 12, wherein the avian is selected from the group consisting of chicken, turkey, quail, pheasant, duck, goose and ratite.

21. The method of Claim 12, wherein the avian is a chicken.

22. The method of claim 12 wherein a polypeptide expressed by the heterologous nucleic acid is delivered to an egg white of a developing avian egg produced by the transgenic avian.

23. A method for the production of a transgenic avian capable of producing a heterologous protein, said method comprising the steps of:

- (a) dissociating a population of cells from an isolated stage X blastoderm, wherein the population of cells includes stage X primordial germ cells and stromal cells;

- 5 (b) incubating the dissociated population of cells in a culture medium for a time sufficient for said stromal cells to sediment from the culture medium;
- (c) isolating from the culture medium a population of cells enriched in stage X primordial germ cells;
- 10 (d) transfecting one or more of said avian stage X primordial germ cells by delivering a heterologous nucleic acid to the population of cells enriched in stage X primordial germ cells, wherein the heterologous nucleic acid encodes a polypeptide to be expressed by a transgenic avian;
- (e) delivering the transfected avian stage X primordial germ cells into a recipient embryo of an avian egg;
- (f) allowing the recipient embryo to hatch as a chick and mature as an adult bird having a heterologous transfected germ cell therein; and
- 15 (g) breeding the adult bird having a heterologous transfected germ cell therein, thereby producing a transgenic progeny bird having the heterologous nucleic acid therein.

24. The method of Claim 23, further comprising the step of breeding the
20 transgenic progeny bird, thereby generating a transgenic progeny bird homozygous for the heterologous nucleic acid.

25. The method of Claim 23, further comprising the step of breeding the
transgenic progeny bird, thereby generating a transgenic progeny bird heterozygous for the
25 heterologous nucleic acid.

26. The method of Claim 23, further comprising the step of expressing the
heterologous polypeptide encoded by the heterologous nucleic acid.

30 27. The method of Claim 26, wherein the heterologous polypeptide is expressed in the serum of a transgenic bird.

28. The method of Claim 26, wherein the expressed heterologous polypeptide is delivered to the white of a developing avian egg produced by a transgenic bird.

35

29. The method of Claim 23, wherein the heterologous nucleic acid comprises an expression cassette having a promoter, a transcription termination sequence and a polypeptide-encoding sequence.

5 30. The method of Claim 29, wherein the expression cassette has a transcription unit encoding a first heterologous polypeptide, and optionally a second heterologous polypeptide, operably linked to a avian specific transcription promoter, a transcription terminator, and optionally an internal ribosome entry site (IRES).

10 31. The method of Claim 26, wherein the transgenic avian expresses a first and a second transgene encoding a first and a second heterologous polypeptide, and wherein the method further comprises the step of combining the first and second heterologous polypeptides, thereby forming a multimeric protein.

15 32. The method of Claim 23, wherein the avian is selected from the group consisting of chicken, turkey, quail, pheasant, duck, goose and ratite.

 33. The method of Claim 23, wherein the avian is a chicken.

20 34. The method of Claim 23, wherein the polypeptide is selected from the group consisting of a cytokine, hormone, enzyme, structural protein, and immunoglobulin.

 35. The method of Claim 34, wherein the cytokine is selected from the group consisting of interferon, interleukin, granulocyte colony-stimulating factor; granulocyte-
25 macrophage colony-stimulating factor; stem cell factor, erythropoietin, thrombopoietin and stem cell factor.

 36. The method of Claim 34, wherein the cytokine is selected from the group consisting of interferon, granulocyte-macrophage colony-stimulating factor and
30 erythropoietin.

 37. The method of Claim 34, wherein the hormone is selected from the group consisting of insulin, insulin-like growth factor, growth hormone, and human growth hormone.
35

38. A transfected avian embryonic stage X primordial germ cell, wherein the stage X primordial germ cell is isolated from an avian egg according to the method of Claim 1, and wherein the stage X primordial germ cell is transfected with a heterologous nucleic acid.

5

39. A transgenic avian producing a heterologous polypeptide in an avian egg, wherein the transgenic avian is produced by transfecting an isolated avian stage X germ cell, delivering the transfected stage X primordial germ cell to a recipient avian embryo for development into a mature avian having a transfected heterologous germ cell therein, and
10 breeding the mature avian with a second avian to generate a transgenic progeny, wherein the progeny comprises at least one heterologous nucleic acid sequence encoding a heterologous polypeptide and wherein the heterologous polypeptide is expressed by the transgenic avian.

15

20

25

30

35

1/2

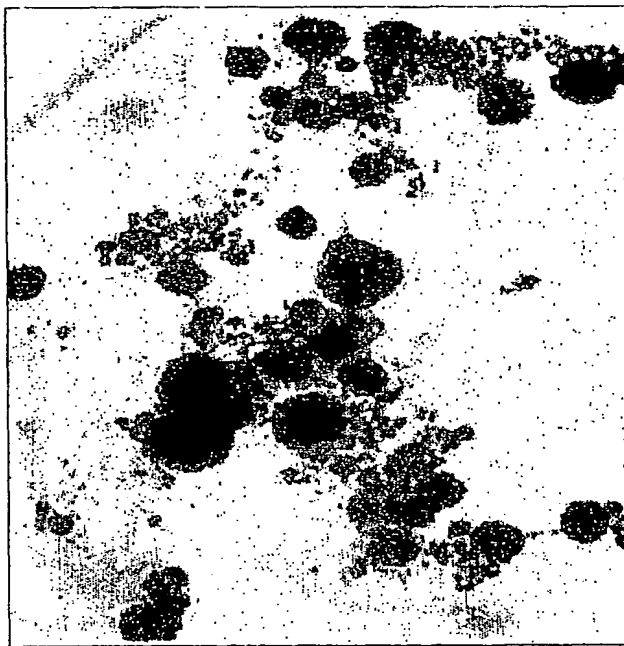


FIG.1B

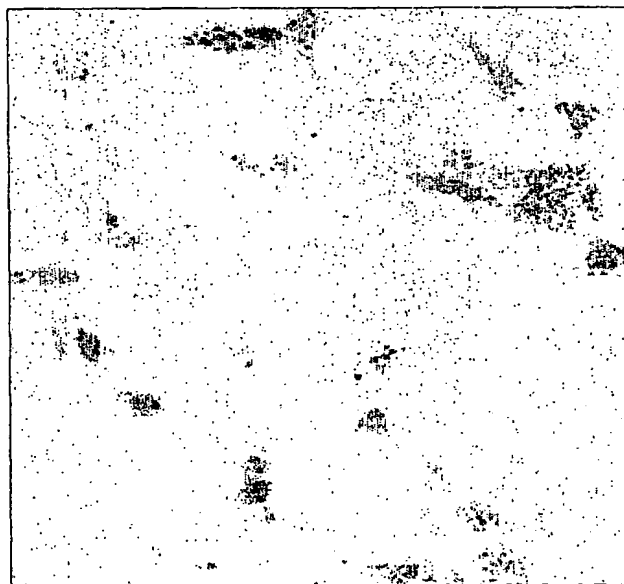


FIG.1A

2/2

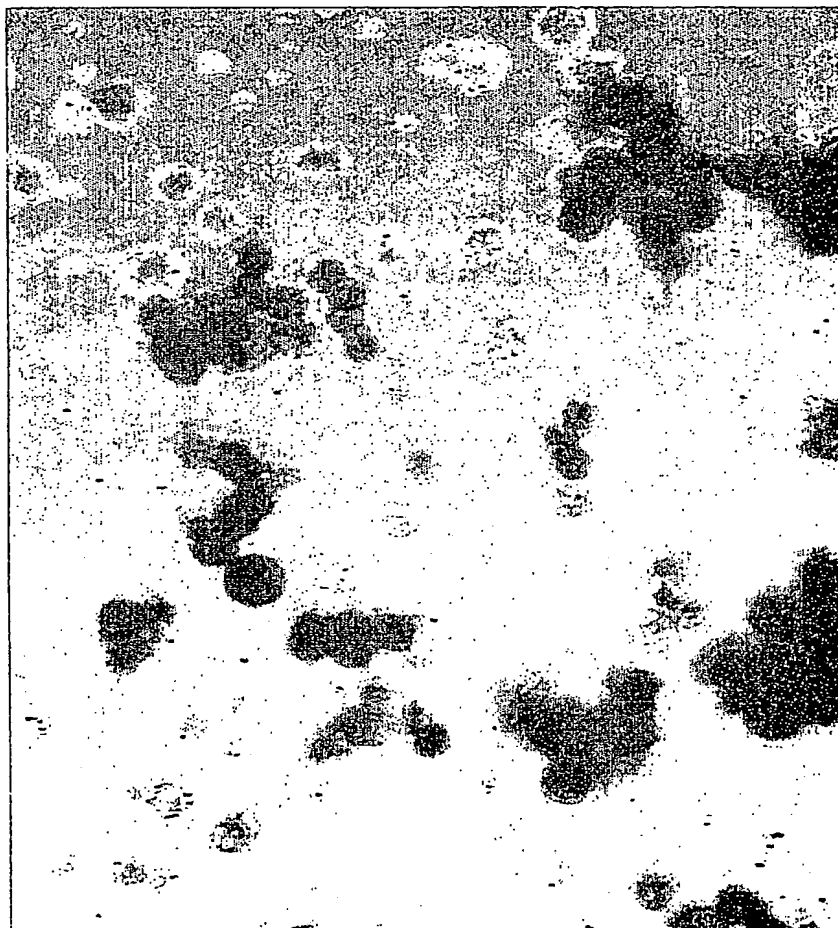


FIG.2